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GROWTH AND DEVELOPMENT OF MERRIAM'S KANGAROO RAT, *Dipodomys merriami*

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INTRODUCTION

No definitive study of growth and development in any species of kangaroo rat has heretofore been published. However, several authors have reported isolated observations and information gained in the course of ecological studies. The most detailed observations are those of Tappe (14) who carefully followed the growth in captivity of four young of one litter of the Tulare kangaroo rat, *D. heermanni,* from the San Joaquin Valley of California. Fitch (7) provides further observations on the development of three young of this species, and also gives weight curves and a proposed growth schedule for wild animals, based on recaptured individuals in the field. Holdenried (9) gives details of weight gains for four young bannertail kangaroo rats, *D. spectabilis,* that were frequently recaptured in the field, one from time of birth, and also monthly average weights and weight ranges for young animals trapped in the field. Bailey (1) gives a few observations and measurements for a captive litter of three *D. spectabilis* from New Mexico. Culbertson (4) followed the growth of one Fresno kangaroo rat, *D. nitratoides,* born and reared in captivity. Doran (6) gives the birth weights of one litter of *D. merriami,* and remarks on development.

The present work is an outgrowth of a field study of populations of desert rodents, emphasizing the most abundant form, *D. merriami.* The principal objective of the work was to provide conclusive information on growth and development of this species, but it was also hoped that it would uncover a method for estimating the age of live kangaroo rats captured in the field. Study of captive animals permitted the making of observations in sufficient numbers and with any desired frequency, which is not true when one must depend on field

recaptures. Data from wild populations provided some check on the equivalence of growth in captive and wild environments. The present paper also reviews literature on growth and development in the genus *Dipodomys*.

The genus *Dipodomys* is of particular interest, since it together with the pocket mice, genus *Perognathus,* forms the bulk of the family Heteromyidae, the only North American family of mammals that can be considered to be basically a desert adapted group. While some species of *Dipodomys* do occur in nonarid habitats, *D. merriami* is to a high degree limited to the Mohave and Sonoran deserts of southwestern United States, Baja California and northern Mexico. *D. merriami* typically burrows in loose alluvial soils, under creosote bushes and other desert shrubs, in areas where there is little or no cover of grasses and herbs to impede its saltatorial locomotion.

Methods and Materials

Details of growth and development were obtained for eight litters of *D. merriami* totaling 22 individuals by the senior author, and for 4 litters totaling 12 individuals by the junior author. Nine of these litters were born in captivity soon after the mother had been live trapped in the desert and brought into the laboratory. Three litters were from females bred in captivity, which is the first reported success in captive breeding of this species (Chew 3). The females bearing these litters were from two desert areas: Alpine Butte, Los Angeles County, 15 miles northeast of Palmdale, California, and Indian Cove, San Bernardino County, nine miles west of Twentynine Palms, California. Both these locations are on the southern edge of the Mohave Desert, about 100 miles apart. Litters from females captured while pregnant were born in February, March and April; litters from females bred in captivity were born in May, August and October.

The data on growth of wild individuals were obtained on animals repeatedly live trapped from a population of *D. merriami* in the Indian Cove area, within the Joshua Tree National Monument, San Bernardino County, California.

Females and their litters were housed in standard wire and sheet metal cages 10 × 11 × 16 inches. An inch or more of clean desert sand was kept in the bottom of the cage. Brown glass bottles and

cotton were provided for nesting purposes. Until the young were at least two weeks old, their cage was covered with brown paper to protect the female from disturbance. Animals of the senior author, both females and weaned young, were in most cases fed a mixed diet composed of 26% rolled oats, 25% rolled barley, 20% meat meal, 10% powdered milk, 11% whole wheat kernels, 2% cod liver oil, 2% sunflower seed, 2% mixed bird seed, 0.5% iodized salt, and 0.5% powdered calcium carbonate. Fresh lettuce was given every other day. Near the end of the study the supply of mixed diet was exhausted, and two litters were given only rolled oats, meat meal, powdered milk, wheat kernels and lettuce. The junior author fed his animals equal amounts of rolled oats and sunflower seeds, providing lettuce daily during the period of rapid growth.

The first few litters were not measured during the first 8 to 12 days of life, for fear that the disturbance might cause the female to abandon her young. However, the later litters were weighed and measured from birth or the day they were first found. The mother kangaroo rats were quite tolerant of this disturbance, immediately retrieved the young, and showed no slackening of maternal instincts. Measurements were taken daily or every other day for the first several weeks, then as growth slowed, the measurements were made only weekly or biweekly.

The following measurements were made on all young animals: body weight, body length, tail length, hind foot length and ear length. On two litters the length and width of the head was also measured. Skull width was measured just behind the eyes. Body length measurements were not made after the animals were about ¾ grown as the struggling of the animal made such measurement too unreliable. Head measurements were made with vernier calipers, other measurements with a flexible millimeter ruler. Linear measurements were read to 0.5 mm., weights to 0.1 gm.

Measurement data were analyzed as in Brody (2). Measurement values were plotted on a logarithmic scale versus age on an arithmetic scale. Linear segments of such a plot indicate periods when growth increments were a constant percentage of previous size. From these linear sections instantaneous growth rates were calculated as:

$$k = \frac{\ln m_2 - \ln m_1}{t_2 - t_1}.$$

The value k is the instantaneous percentage rate of growth for the unit of time in which t_2 and t_1 are expressed; ln m_2 and ln m_1 are natural logarithms of the measurements made at t_1 and t_2.

Observations and Discussion

General Development. Structure. Newborn *D. merriami* are quite naked. The skin is pink, thin and transparent. The abdominal organs are visible through the venter, and the dorsal sutures and blood vessels of the skull are particularly marked. The snout areas containing the bases of the vibrissae are very swollen. The vibrissae are about 5 mm. long at birth and rapidly lengthen to 17-21 mm. at 11 days.

On the 3rd day a pattern of black pigmentation begins to appear in the skin and gradually fills in those areas that will be brown in the adult. Pigmentation begins on the back and then develops, in sequence, on the sides, dorsal tail stripe, sole of hind foot, head, flanks and ventral tail stripe. The area of the dorsal skin gland is obvious as an unpigmented pink oval spot in the middle of the back just posterior to the scapulae. By the 5th or 6th day the head is sufficiently pigmented that the dorsal sutures and blood vessels are no longer visible.

White hairs begin to appear on the flanks on the 5th day. Short brown hairs begin to appear in the pigmented areas on the 6th or 7th day, and by the 8th day the brown begins to mask the dark grey of the skin pigments. By the 9th day the brown color is quite distinct, particularly on the back, less so on head, dorsal tail stripe and flanks. The ventral tail stripe and soles of hind feet do not become distinctly brown until the 10th day. The skin gland area does not become haired, but is now darkly pigmented and is obvious as a black oval in the midst of the brown fur. By the 11th day the mammae have become very distinct; the insides of the thighs and the ventral surface in general is still sparcely haired. By the 14th day the animal is fully furred, and the dorsal skin gland is no longer visible, being overlaid by brown hairs now 3 to 4 mm. long. The color pattern is as in the adult (figure 1), but the brown is a rich chocolate brown, much darker than in the adult, contrasting strongly with the white areas. As the individual ages, the fur becomes quite pale brown, and then darkens into the adult shade of brown.

The ears grow, but the meatus remains closed until the 8th to 10th

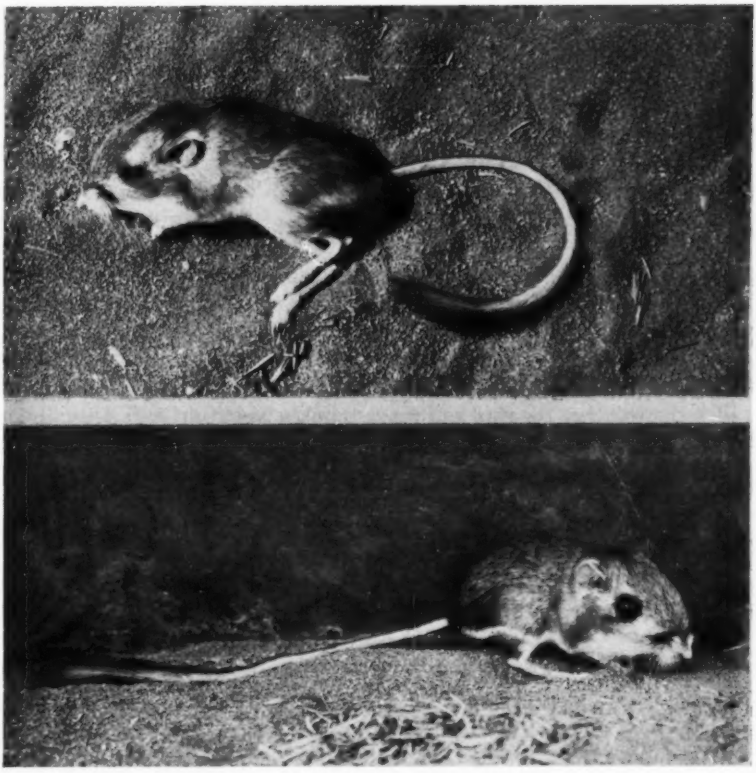

FIGURE 1
Photographs of adult *Dipodomys merriami*. Dark fur is brownish. Note characteristic white markings: stripe on flank, patches above eye and behind ear.

day. The eyes are open, on the average, by the 12th or 13th day; the earliest opening was on the 11th day, the latest on the 15th. At first the eyelids are parted only by a narrow slit, and even when they are fully retracted the eyes are at first obviously uncoordinated.

The incisors break through the gums as early as the 2nd day, and are 1 mm. long by the 8th day.

The sex of the young rats could be determined with fair accuracy by the 11th day. The vulva first opened in young females on the

24th to 33rd days. Swelling and crusting of the vulva was first seen on the 34th to 37th days. Males appeared to have scrotal testes by the 85th day. However, animals autopsied at this age had small testes and the scrotal protuberance appeared to be primarily epididymis rather than descended testes.

Behavior. Newborn rats can weakly crawl about. They make an immediate though clumsy righting response when turned over. By the 2nd day they move about very deliberately with the primitive tetrapod sequence of leg movements. On the third day, if litter mates are separated in a small pan of sand, they will move slowly around the edge of the pan until they touch each other, and then by a laborious turning movement, pivoting on the right rear leg, they snuggle closely together. The highly developed vibrissae are obviously important in this huddling response. For the first week the tail is semiprehensile.

By the 9th day the rats can move quickly about on four feet. They will now run out of the nest when disturbed by a sound, which response is coincident with the opening of the ears. By the 11th day the kangaroo rats are beginning to hop on their hind feet, and can leap vertically 3-4 inches. This jumping ability increases to 6-8 inches at 13 days and 12 inches at 15 days.

At 11 days young animals will scratch and wash with their tongue when sprinkled with water. Not until the 17th day do they make clumsy attempts at rolling and stretching in the sand when sprinkled. By the 19th to 22nd day they can expertly execute the sand washing and tail preening characteristic of *Dipodomys*. On the 22nd day they also first show the characteristic rotary motion of the body when held suspended by the tail.

Young kangaroo rats try to eat lettuce at 9 days, and are probably fully weaned by the 17th to 22nd day. As early as the 15th day they begin putting objects in their cheek pouches, but at first are not discriminatory and take bits of wood, rabbit droppings and small stones. On the 19th to 22nd days they begin the characteristic progressive digging through sand, possibly a food searching act. By the 23rd day they show discrimination in pouching only seeds, and also begin caching food in the sand.

These young *D. merriami* are more precocious than the three observed by Doran (6) which did not show hair until the 6th day, opening of ears 16th day, opening of eyes 21st day, and eating solid

food 32nd day. However, their general development is very similar to that of *D. heermanni* as observed by Tappe (14, p. 136) except that in *D. heermanni* the eyes and ears did not open until the 14-16th day and 16th day respectively and weaning extended from the 17th to 25th day. Fitch (7, p. 27) also judged that *D. heermanni* was weaned by the 25th day. Culbertson (4, p. 201) found that *D. nitratoides* has its eyes open by the 13-14th day. Bailey (1, p. 256) found that *D. spectabilis* has its eyes open by the 14th day, incisors through gums on 11th day, and is weaned by the 25th day.

In its speed of development, *Dipodomys* is similar to most other small rodents for which data are available. It lies between the extremes of the very precocious development of the cotton rat, *Sigmodon hispidus,* and the relatively slow development of the wood rat, *Neotoma albigula. Sigmodon* is very well developed at birth, is capable of running about actively at birth, and has its eyes open the first day (Svihla 12, p. 352-3). *Neotoma* does not have its eyes open until the 15th to 19th days and weaning is not complete until the 42nd to 47th day (Richardson 11, p. 133).

Increase in body weight. If weight is plotted on a logarithmic scale, as in figure 2, it is obvious that there are several phases of growth during which the percentage of increase per day is constant. The first three phases, as in figure 2, are usually very linear. During the fourth phase body weight oscillates, but around an increasing slope. The rate of growth in the fourth phase was sensitive to the adequacy of the diet. Litters fed on the full mixed diet described under methods grew as in figure 2. Two litters that were fed the more limited diet grew normally during the first three phases, presumably carried along for a short time after weaning by the nutritional impetus from the previous milk diet, but then growth practically ceased, so that phase 4 was nearly horizontal.

Figure 3 gives composite graphs for the 8 and 4 litters studied by the two authors, omitting the later phase of the two litters that were obviously not adequately fed. The duration and slope of each phase is an average for all the litters of each author. Weight increase is accomplished in five phases after birth. Instantaneous growth rates are indicated by the k values above each segment of the curve. For example, during the first growth phase after birth, weight increased 13 percent per day; in the fifth phase, adult weight is slowly approached at the rate of 0.33 percent per day.

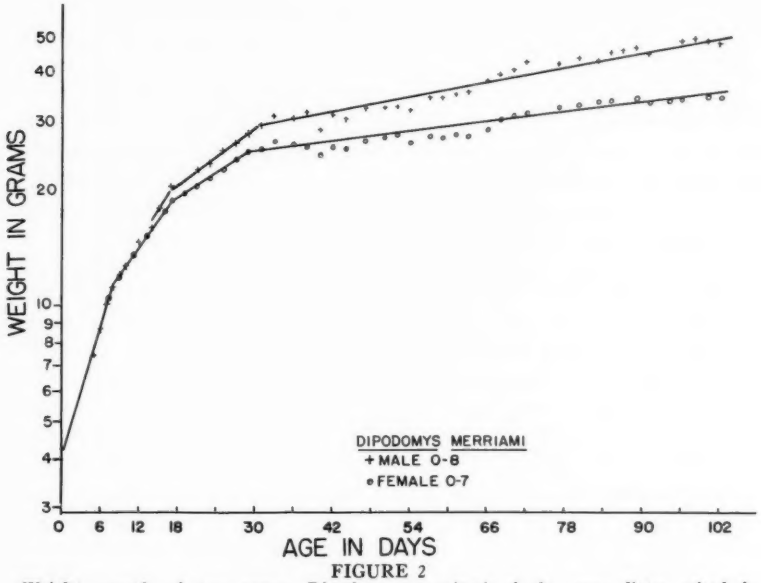

FIGURE 2

Weight growth of two young *Dipodomys merriami* of the same litter raised in captivity.

Simply for purposes of comparison with later growth phases, fetal growth is crudely estimated (as 32.9% per day) from the known birth weights and approximate gestation time. In the absence of actual measurements, linear increase is assumed from fertilization to birth, though there is probably a considerable period in early gestation when this is not true. From the females that were bred in captivity, length of gestation is estimated as 33 ± 2 days. Adult females were placed with males when the first swelling and opening of the vulva was noted, and they were left together four or five days until regression or plugging of the vulva was noted. Since copulation most probably occurs early in this sequence of vulvar changes, gestation length is more likely greater than 33 days than less.

Below the curve are indicated several important events in the life of the individual. Several coincidences are apparent. The first inflection after birth occurs just after the opening of the eyes, when there

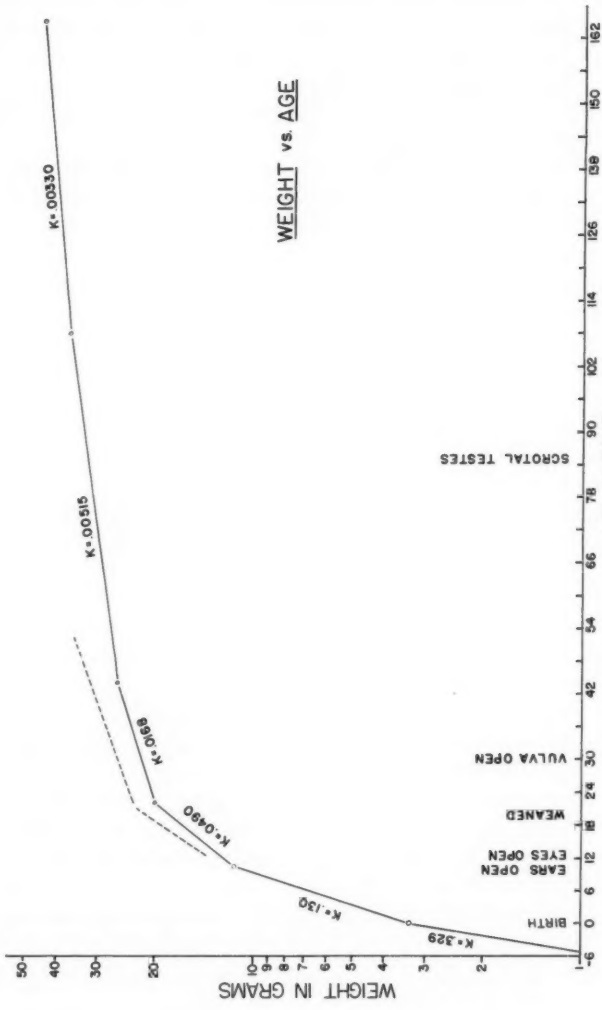

FIGURE 3

Composite plots of data for weight growth of 12 litters of *Dipodomys merriami* raised in captivity. Solid line is for 8 litters of senior author. Values with segments of plot are instantaneous growth rates; value x 100 = percentage increase per day during that phase of growth. Broken line is for 4 litters of junior author.

is an increase in activity which probably reduces the energy that can go into growth. The second inflection follows several days after the estimated time of full weaning. The change in diet can also be expected to alter the rate of growth.

Inflections of growth at the times of weaning and opening of eyes are also present in other wild rodents for which sufficient data are available: the deer mouse, *Peromyscus* spp. (McCabe and Blanchard 10, Svihla 13), wood rat, *Neotoma albigula* (Richardson 11), and pine mouse, *Pitymys pinetorum* (Hamilton 8).

Figure 4 gives a comparative plotting of data available for other species of *Dipodomys*. Only the data of Tappe (14, p. 140) for *D. heermanni* were taken with sufficient frequency to be susceptible to analysis as for *D. merriami*. The first phase of growth of *D. heermanni* was slower and longer lasting than for *D. merriami*, however later growth was more rapid. The break at 16-18 days for *D. heermanni* coincides with the opening of the eyes and ears and the beginning of weaning. It is guessed that the break at 30-36 days represents a nutritional irregularity.

Also given in figure 4 are data on the growth of white rats (after Brody 2, figs. 16:10 and 16:12); there is considerable similarity between the growth of white rats and *D. merriami*, especially in the first several phases. Fetal growth of the white rat occurred at an average rate of 53 percent per day, as compared to 32.9 percent for *D. merriami*, however the gestation period of the white rat is only 22 days as compared to 33 ± 2 for *D. merriami*.

It is not possible to compare the first two phases of growth of captive and wild *Dipodomys*, since these phases are completed before the young animals begin to leave their burrows to forage. The smallest *D. merriami* live-trapped in the present study were 20.0, 20.5, 23.8, 25.8 and 26.3 gm. in weight. Assuming the rate of growth in the laboratory, these kangaroo rats were already in their third phase of growth and 24 to 43 days old. Fitch (7, p. 27) estimated that *D. heermanni* may begin to forage as early as 4 weeks, while Tappe (14, p. 144) estimated this species leaves its burrows for the first time at about 6 weeks of age. *D. nitratoides* begins to forage as early as 5 weeks (Culbertson 4, p. 202).

Data are available for the growth of subadults in the field (fourth and fifth phases of laboratory growth). Average weights of *D.*

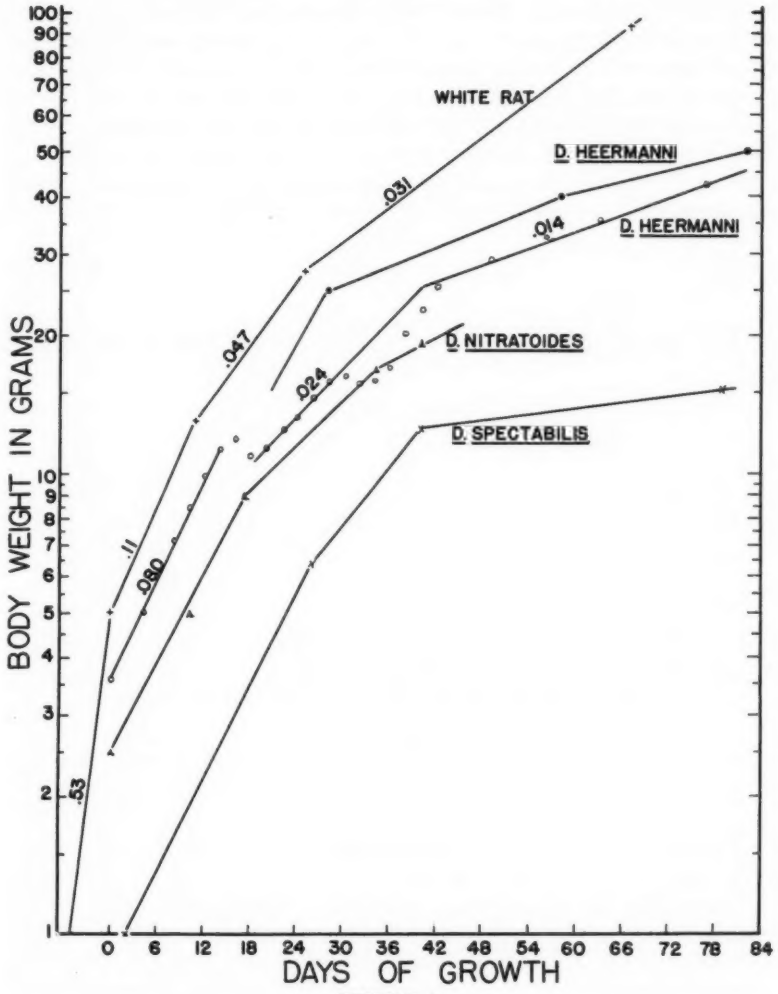

FIGURE 4

Semilogarithmic plot of growth data for the white rat and several species of kangaroo rats. Sources of data: white rat, Brody (2); *D. heermanni,* solid circle plot, Fitch (7), open circle plot, Tappe (14); *D. nitratoides,* Culbertson (4); *D. spectabilis,* Bailey (1). Values above segments of plot are instantaneous growth rates.

merriami recaptured during four consecutive trapping periods November 30 to February 15, 1957 (figure 5) show a weight increase of 0.22 percent per day for males and 0.25 percent for females, which is less than the 0.33 percent for laboratory reared animals in the same weight range. After February weight declined. Field weight, of course, is highly sensitive to food supply and environmental stress, and no equality of comparison is certain. Growth apparently was somewhat more rapid in the laboratory.

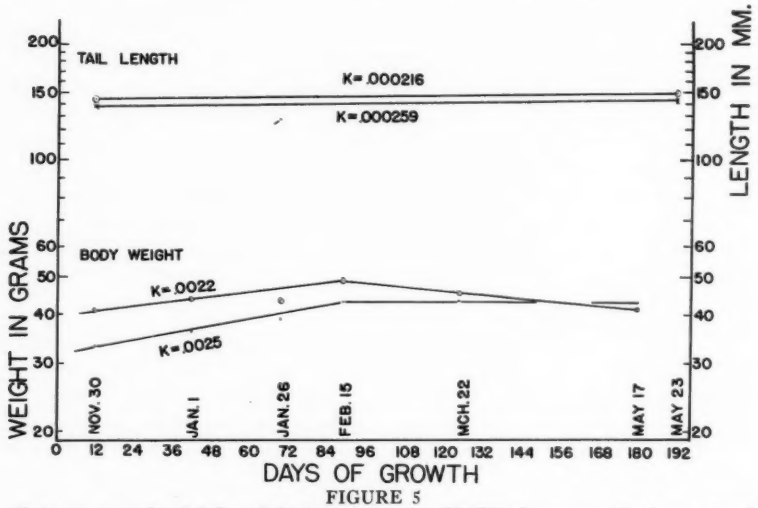

FIGURE 5

Data on growth of tail and body weight of wild *Dipodomys merriami* recaptured four to six times in six trapping periods from November 30, 1956 to May 23, 1957. Values above segments of plot are instantaneous growth rates.

Figure 4 gives for *D. heermanni* both the data of Tappe (14, p. 141) on captive animals and that of Fitch (7, p. 27) for wild individuals. The growth schedule proposed by Fitch is somewhat more rapid than that of the captive animals. The data of Holdenried (9, p. 342-5) for *D. spectabilis* are entirely for field animals. The four young animals that were captured repeatedly showed a terminal rate of growth similar to that indicated simply by the increasing average weight of subadult animals captured each month in the field. Terminal growth of wild animals was 0.157 percent per day for males and

0.198 percent for females, somewhat slower than field growth for *D. merriami* in the present study.

Figure 6 shows the rate at which adult weight is achieved in *D. merriami;* 25 percent of weight growth is completed by the 12th day, 50 percent by 35th day, 75 percent by 84th day, 90 percent by 140th day, and 100 percent by 203rd day. Estimates from data of other authors as to completion of weight growth are: *D. spectabilis* about one year (Holdenried 9), captive young reached mother's weight in 4 months (Bailey 1, p. 256); *D. heermanni* 90 to 102 days (Fitch 7, Tappe 14).

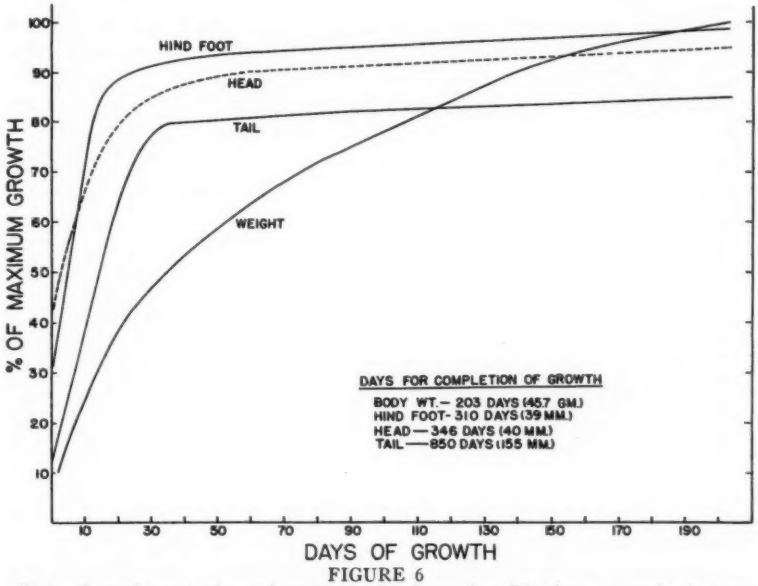

FIGURE 6

Rate of attainment of maximum growth of captive *Dipodomys merriami;* composite of data for 22 individuals of 8 litters of senior author.

The lengths of time for completion of weight and linear growth in *D. merriami* are considerably greater than most of the values reported in the literature for other species of kangaroo rats and for rodents in general, which, however, are usually stated as ". . . days to reach adult size." As emphasized by McCabe and Blanchard

(10, p. 46) the difficulty lies in establishing the maximum value toward which growth is proceeding. Often "adult" measurements are based on a relatively small series of animals, judged as mature on the basis of their general size and appearance. Such animals are certainly sexually mature, but growth may still be continuing. In agreement with the present findings, Dice (5) found that deer mice, *Peromyscus,* continue to grow at a slow rate even after two years. In the present study, maximum size or 100 percent size was taken as the average of the top 6 to 12 measurements recorded from field animals.

Birth weights of *D. merriami* were reliably obtained for 9 litters. Birth weights averaged 3.04 grams and ranged from 2.2 gm. to 4.6 gm. The heaviest individuals were those of litters of two from females bred in captivity, having average weights of 3.9 and 4.6 gm. respectively. Litters born to females captured when pregnant averaged: 2.2 gm. (2 young), 2.5 (3), 2.8 (4), 3.25 (4), 3.4 (3), 4.0 (2). Litters that did not survive had average weights of: 1.25 (2 young), 2.2 (3), 2.5 (3) and 3.2 (1). The smaller individuals were undoubtedly born prematurely.

Birth weights for other species of *Dipodomys* are:

Species	Av. Wt. (Range)	Number Weighed	Adult Weight	Source
D. merriami	3.5 (3.2-3.8)	3	41.5	Doran (6)
D. nitratoides	2.5 (1.5-3.5)	7	31.2	Culbertson (4, p.201)
D. heermanni	3.65(3.4-3.9)	4	—	Tappe (14, p.136)
D. heermanni	"as high as 5 gm."	—	60.0	Fitch (7, p.26)
D. spectabilis	7.7 (6.0-8.7)	7	139	Holdenried (9, p.342)
D. spectabilis	7.8 (5.3-9.3)	—	—	Bailey (1, p.255)

There is the expected relationship between weight of newborn and weight of adult.

Increase in linear dimensions. Figure 7 presents a composite picture of growth of the dimensions usually recorded by mammalogists. Linear measurements also show phasic growth, with periods of constant percentage increase and then rather abrupt changes to lower rates of growth. Except for hind foot length, the plotted measurements agree in having their first growth inflection at about the 14th day and the second inflection on the 28th to 30th day. The causes of these inflections are not certain, however they occur just a few

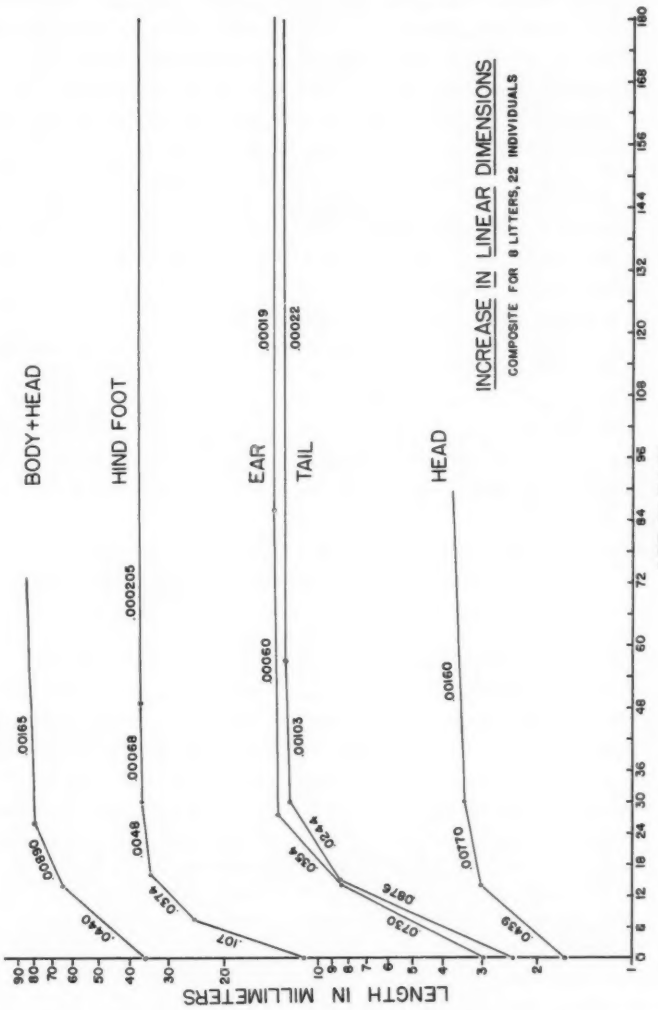

FIGURE 7

Composite of growth of linear dimensions of 8 litters of *Dipodomys merriami* raised in captivity. Values above segments of curve are instantaneous growth rate. For tail length, multiply scale by 10.

days after the weight increase inflections that coincide with the opening of the eyes and weaning. Usually only four growth phases were apparent as compared with five phases in weight increase.

The hind foot grew more rapidly than the other dimensions, completing two phases of growth in the same time that the other dimensions completed one phase. To human judgment it is obvious that the hind feet are one of the most necessary developments of a kangaroo rat. Kangaroo rats evade predators by long jumps made in erratic directions (a type of locomotion that is possible only in an open situation with much bare ground, such as a desert habitat), hence the logic in the evolution of a rapidly growing hind foot. The young animals are quite adept in the use of these hind feet in saltatorial locomotion as early as 15 days of age, which is considerably before the time they normally leave the nest.

The rapid growth of the hind foot is more obvious in figure 6, where it is shown that 75 percent of maximum growth is attained in 10 days, 90 percent in about 25 days. The tail reaches 80 percent of total length in about 35 days, but then the rate of completion of growth abruptly declines.

The data of Tappe for *D. heermanni* (14, p. 140) plotted in figure 8 also show phasic growth, which is in general similar to that of *D. merriami*. There is no general correspondence of the time of the first growth inflection of the different dimensions. The striking contrast with *D. merriami* is that the ear, rather than the hind foot, is the fastest growing dimension. The body grows more rapidly than in *D. merriami;* the tail growth of the two species is about equal in the first two phases, but later growth is more rapid in *D. heermanni.*

Estimation of age of live individuals. Tail length is apparently the only usable measurement for estimating the actual age of a live individual after 1 to 2 months. Tail length is the only dimension that continues to increase in reliably measurable amounts throughout the natural life span of the kangaroo rat. From laboratory data and field data (figure 5) it appears that the tail continues to increase at the rate of about 0.66 percent per month after the age of 2 months. At this rate, beginning with an average tail length of 134 mm. at 6 months, it would take 850 days or 2 years 4 months for a kangaroo rat to attain the longer tail lengths observed in the field, 160 mm. for male *D. merriami* and 150 mm. for females.

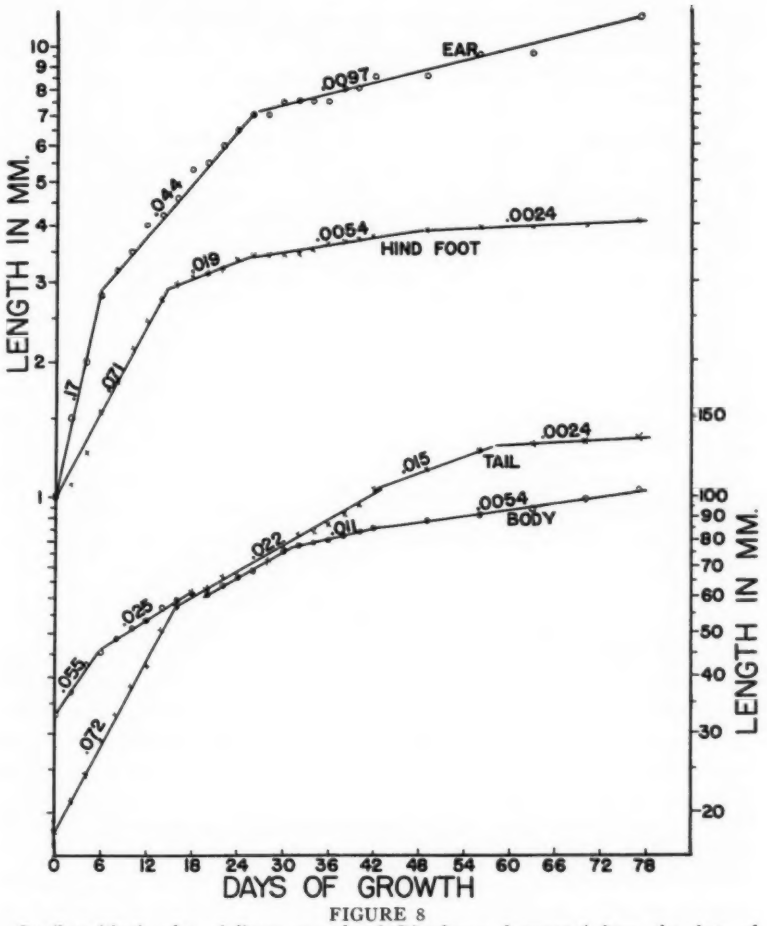

FIGURE 8

Semilogarithmic plot of linear growth of *Dipodomys heermanni* from the data of Tappe (14).

Ear, hind foot and body length are not usable for aging because after one month their absolute increments are so small as to be unreliably measured, especially on living animals. Weight is not useful, since it undergoes negative trends during adverse seasons, as shown in

figure 5. Tail length continued to increase during these same months. Use of a linear measurement for aging has the advantage that it does not undergo negative changes, though its growth may be slowed during adverse seasons.

The following is suggested as a possible procedure for estimating the age of live *Dipodomys*: (1) Establishment of rate of tail growth of wild individuals on basis of a reasonable sample of individuals recaptured over 8-12 months. (2) Calculation of a tail length-age scale, using the determined rate of growth and a starting point such as 134 mm. at 6 months for *D. merriami*. (3) Placing individuals for each trapping period into these tail length-age groups.

While individual variation in the rate of tail growth would make for quite a few errors in placement of individuals, these errors should be reasonably equal in negative and positive directions, so that the overall classification of an adequate sample of animals should be reliable.

For the field populations in the present study, assuming that an average tail length of 134 mm. is reached by age of 6 months, and that the rate of increase is then 0.66 percent per month, tail lengths and corresponding ages would be: 134 mm., 6 mo.; 138 mm., 10 mo.; 142 mm., 16 mo.; 146 mm., 20 mo.; 150 mm., 24 mo.; 154 mm., 28 mo. Figure 9 shows a plotting of data for populations of November 1956 and September 1957 into tail length groups. The concentrations of individuals into certain groups may indicate the breeding seasons of *D. merriami*. If these concentrations are translated into ages according to the above scale, most of the estimated birth dates are at reasonable times of the year, times during which reproduction was observed to occur in Indian Cove. The occurrence of age group concentrations, having their origins at breeding seasons, lends credence to this method of aging. However, the number of individuals over 1½ years of age in this analysis is suspect in terms of data from recapture studies on wild populations of *D. spectabilis* in which marked individuals disappeared on the average of 4 months after marking (Holdenried 9, p. 339).

Unfortunately, in the present study the value of tail measurements was not realized until late, and insufficient data were gathered on this to allow a decisive evaluation of aging by tail lengths.

Head length and width were measured on three litters in the hope

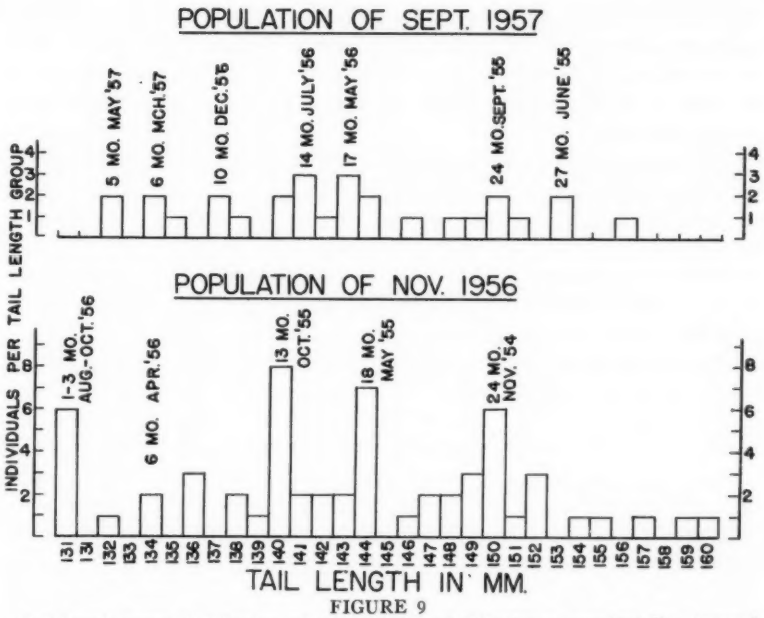
POPULATION OF SEPT. 1957
5 MO. MAY '57
6 MO. MCH '57
10 MO. DEC. '55
14 MO. JULY '56
17 MO. MAY '56
24 MO. SEPT. '55
27 MO. JUNE '55
POPULATION OF NOV. 1956
1-3 MO. AUG.-OCT. '56
6 MO. APR. '56
13 MO. OCT. '55
18 MO. MAY '55
24 MO. NOV. '54
INDIVIDUALS PER TAIL LENGTH GROUP
TAIL LENGTH IN MM.

FIGURE 9

Tail length group distribution of two samples of *Dipodomys merriami* live trapped at Indian Cove, Joshua Tree National Monument, California. Estimates of age and birth date are given for the larger groupings.

that changing proportions of the skull might be an age criterion. It was found that the length/width ratio changed from 1.431 at 1 week, to 1.438 at 2 weeks, to 1.512 at 3 weeks, to 1.533 at 4 weeks but then leveled off at 1.601 at 45 days and was not useful for aging after that time. There is also difficulty in measuring head dimensions on live animals with sufficient accuracy for determining ratios such as length: width.

Summary and Conclusions

Data are presented on the growth and development of 34 individuals of 12 litters of *Dipodomys merriami* reared in captivity. These data are compared with those in the literature for this and other species.

New born kangaroo rats are naked; pigmentation appears by the

3rd day and the first hair by 5th or 6th day. Vibrissae are well developed at birth and are the initially important sense organ. Ears open on the 8th to 10th days, eyes on 11th to 15th days. The vagina opens on 24th to 33rd days and males appear to have scrotal testes as early as the 85th day.

The young can crawl feebly at birth; quadrupedal locomotion is well developed by the 9th day and hopping locomotion occurs by the 11th day. The characteristic behaviors of sand washing and pouching and caching of food are well developed by the 19th to 23rd days. Weaning is completed by the 17th to 22nd day and wild young begin to leave their nest to forage by the 24th to 34th day.

Weight increase is accomplished in five phases, each of which has a relatively constant absolute growth rate, ranging from 13%/day in the first phase after birth to 0.33%/day in the final phase. Fetal growth is estimated to occur at a rate of 32.5%/day. Growth inflections occur at the time of opening of the eyes and at weaning.

The tail, body, head, hind foot and ear also show phasic growth, which is usually completed in four phases. The hind foot grows considerably faster than the other dimensions that were measured, reaching 90% of maximum size in 24 days. This is obviously correlated with the overdevelopment of the hind appendages in this genus, and the dependence on saltatorial locomotion.

The tail reaches 85% of maximum length in 35 days and then grows slowly for an estimated 28 months before reaching maximum tail lengths observed in the field.

A method is suggested for estimating the ages of live individuals captured in the field, based on growth of tail.

Acknowledgements

The work of this paper represents a joint effort of both authors working independently and together in the accumulation of the data on which this paper is based. The senior author outlined the scope of the paper and wrote it essentially as it appears. The authors gratefully acknowledge Mr. Samuel King, Superintendent of Joshua Tree National Monument when this work was begun, for granting permission to carry out live trapping of rodents in a study area within the Monument. The work has also been aided and encouraged by Naturalist Bruce Black. The investigation by the junior author was sup-

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GROWTH AND SURVIVAL OF BIRDS UNDER CHRONIC ACCELERATION[1]

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THE biological effect of accelerative forces developed by circular motion was recognized in 1921, when aviators experienced a dimming of vision during high-speed turns (Poppen and Drinker, 1950-51). The physiological changes that occur during acute exposure to centrifugal forces, intensively investigated because of their importance in aviation medicine, are currently well described (Stacy et al., 1955). However, physiological changes resulting from prolonged exposure to such forces (amounting to a simulated change in gravity) have received much less attention. Matthews (1953) reported the effects in rats of a year's exposure to 3 G (3 earth gravities; 3 x 980 dynes). Fruit fly larvae raised under very strong accelerative forces (1630-3100 G) exhibited a growth regression that was proportional to field strength (Wunder, 1955). The growth of wheat plants has also been studied under moderate accelerative forces (Gray and Edwards, 1955; Edwards and Gray, 1956).

This report deals with the influence of prolonged exposure to centrifugal forces (i.e., chronic acceleration stress) on the growth and survival of chickens and turkeys, which, being bipeds, are posturally similar to man.

METHOD

Acceleration was provided by an animal centrifuge capable of producing accelerative forces up to 6 G; its operation and constructional details will be reported elsewhere. The birds were kept in cages connected to the centrifuge arms by pivots so that the accelerative force field (the resultant of gravity and centrifugal force) was perpendicular to the cage floor. After a preliminary period of two or three days, to

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accustom the birds to the cage, centrifugation started and was continuous, except for the few minutes a day required to replenish feed and remove manure. At weekly intervals the birds were removed from the centrifuge for a longer period, usually about half an hour, for determining body weight and measuring various physiological phenomena. Control birds were kept in the same room, and under similar conditions of air movement, temperature, illumination (continuous), population density, etc. After the centrifugation period, the birds were raised to maturity in individual cages.

In a preliminary experiment (trial A) it appeared the chicks could withstand accelerative forces up to 2.5 G for at least 15 weeks, and perhaps more-or-less indefinitely. The results from this trial are not reported further, since the control group, which was housed in a separate area, suffered an outbreak of respiratory disease. Subsequently, two types of centrifugation trials were conducted: (1) long term exposure, about six months, to a moderate force, 2.5 G; and (2) shorter exposure, about seven weeks, to a more intense acceleration, starting at 1.5 G and increasing to 4 G by weekly increments of 0.5 G. Several sorts of birds were employed all from stocks previously unexposed to accelerative forces: a cross-breed (New Hampshire vs. Single Comb White Leghorn) was used in the longer experiment and S. C. White Leghorns, New Hampshires, and turkeys (Broad-breasted Bronze) in the shorter experiments. Centrifugation was started when the birds were 14-16 days old, except for a group of S. C. White Leghorns (trial E) that was brooded normally to six weeks of age before experimentation. No heat beyond that from the lights in the cages was provided for either experimental or control birds. However, room temperatures rarely fell below 70°F, which, though somewhat lower than standard brooding practice, does not appear to affect growth significantly (Winchester and Kleiber, 1938; Barott and Pringle, 1950). In one experiment (trial F) an extra control group raised in a brooder under usual conditions showed the same growth as the unheated control group.

The growth characteristics of the New Hampshire and White Leghorn chickens used in these experiments were comparable to those of commercial strains (Rosenberg et al., 1949). The turkeys showed a somewhat slower growth rate than indicated by commercial production standards (Scott, 1959), although their growth was similar to that of other groups of this strain raised in the departmental plant.

RESULTS

Prolonged centrifugation resulted in severe mortality, whereas little or none was encountered in the controls (Table I). The body size and growth characteristics of birds that died during centrifugation were examined for any correlation with mortality. In the younger birds, for which centrifugation started at 14-16 days age, those that died early (i.e., in the first week) were generally smaller than those that

TABLE I

MORTALITY RATE DURING PROLONGED CENTRIFUGATION

The number of birds dying is presented as percent of the original group (n). When birds were sacrificed for anatomic data or to reduce the size of the group, their number was divided between those dying and surviving on the basis of later mortality.

	Control				Experimental			
	♂		♀		♂		♀	
Trial desig.	(n)	Mort.%	(n)	Mort.%	(n)	Mort.%	(n)	Mort.%
I. Short-term (6-7 weeks) exposure to high intensity (1.5-4 G) acceleration:								
A. White Leghorn chicks, two weeks old at start:								
B	(72)	16.5%	(67)	6.6%	(73)	64.0%	(74)	72.7%
E	(3)	0	(3)	0	(3)	66.7%	(9)	78.0%
F	(18)	0	(16)	0	(58)	60.4%	(56)	72.1%
Mean		12.8%		5.1%		62.5%		72.9%
B. White Leghorn chicks, six weeks old at start:								
E	(11)	0	(9)	0	(7)	85.8%	(12)	75.0%
C. New Hampshire chicks, two weeks old at start:								
D	(19)	15.8%	(19)	10.5%	(19)	89.5%	(19)	89.5%
D. Turkey poults, two weeks old at start:								
D	(12)	16.7%	(12)	0	(12)	75.0%	(12)	66.7%
II. Long-term (24 weeks) exposure to moderate acceleration; New Hampshire vs. White Leghorn chicks, two weeks old at start:								
C	(42)	14.3%	(47)	51.8%*	(49)	46.3%	(77)	35.8%

* Most of the control mortality was from visceral lymphomatosis, to which cross-breeds are quite susceptible. Mortality from other causes was negligible. There is no obvious explanation for the absence of this disease in the experimental birds.

survived to the end of centrifugation. It is doubtful if initial body size itself is the factor; probably this is merely an index of metabolic capacity. Although small animals with a high specific surface might be more susceptible to chilling, and also to injury in huddling, these factors did not appear to affect survival. These early deaths appeared to involve very susceptible individuals that became immobile and

moribund from even a few days of exposure to moderate accelerative forces. They did not appear to suffer any severe or permanent damage, since they would recover rapidly (within 24 hours) at normal gravity; however, when left in the centrifuge, such animals died in a few hours. Such individuals also were trampled by other birds, and, although the resulting trauma may have contributed to their death, they would not have survived even if isolated.

Younger birds that died late in the experiment, i.e., after 3-4 weeks or more of centrifugation, did not differ from the survivors in size or growth rate at the start of centrifugation, nor were any changes in these characteristics always apparent in the week preceding centrifugation death. However, the survivors were generally not the largest animals at the start of the experiment.

In the group of birds that were brooded normally and six weeks old at the start of centrifugation (series E), a body size close to the mean for the group appeared to enhance survival. When deviation in initial weight from the group mean was compared with survival, the following relation was obtained:

$$\text{Survival} = 165 - 6\ (\triangle\ \%\text{W})$$

Where: Survival is the integral of acceleration (i.e., G-days) up to the time of death;

$$\triangle\ \%\text{W is calculated as: } \frac{\text{W} - \overline{\text{W}}}{\overline{\text{W}}}\ 100$$

This relation fits the survival of the most susceptible and resistant birds quite well, but some intermediates deviated, dying sooner than predicted (Fig. 1). Other mathematical treatment of the data (i.e., semi-logarithmic or exponential) does not improve its conformance. Extrapolations of this relationship indicate that animals with weights deviating more than 27.5% from the mean would be non-viable in the centrifuge, and that maximum survival under such conditions would be 165 G-days (i.e., about a week at 4 G beyond the termination of the experiment). However, these implications relate only to the particular acceleration schedule (7 weeks at 1.5-4 G) and probably are not generally applicable. The assumption that G-days are equivalent in prolonged centrifugation is obviously incorrect (viz., in trial C birds survived 24 weeks exposure to 2.5 G, or 420 G-days; and in acute exposure, the lethal limit for chickens is reported to be 40-45 G-

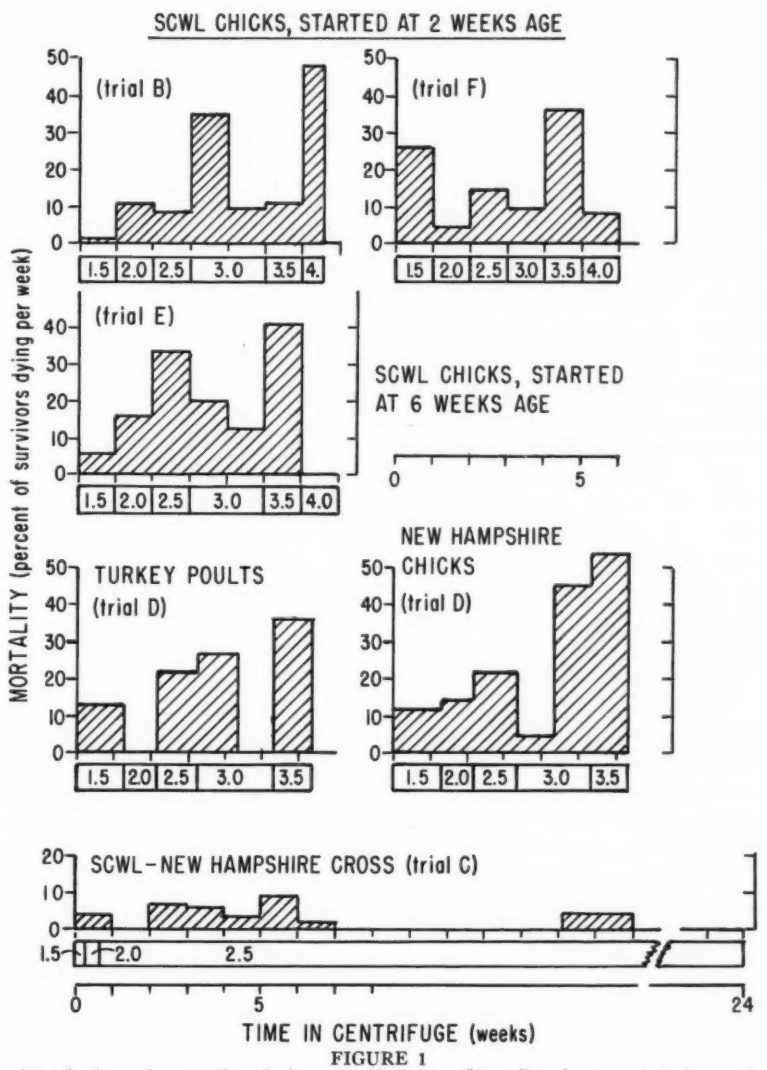
SCWL CHICKS, STARTED AT 2 WEEKS AGE
(trial B)
(trial F)
(trial E)
SCWL CHICKS, STARTED AT 6 WEEKS AGE
TURKEY POULTS (trial D)
NEW HAMPSHIRE CHICKS (trial D)
SCWL-NEW HAMPSHIRE CROSS (trial C)
MORTALITY (percent of survivors dying per week)
TIME IN CENTRIFUGE (weeks)

FIGURE 1

Distribution of mortality during centrifugation. Mortality is presented for each increment of accelerative force as percent deaths per week of birds starting that period. The acceleration schedule is indicated for each trial.

minutes, Britton et al., 1946). However, at this time there is not enough information to evaluate the time-intensity relationship of prolonged exposure to various accelerative forces. This finding is quite different from the lethal limit of rats exposed to 3.5-34 G for 1.5-60 minutes, in which a uniform response followed equal "g x t" treatments (Britton et al., 1946).

The equal lethal affect of positive and negative deviations from mean size indicates that at least two types of factors are involved in acceleration-caused death. Smaller animals may be those with a low metabolic power; hence, their survival might be limited by biological factors. Larger animals, however, would be more susceptible to effects arising from increased fluid density (i.e., their vascular systems would present longer hydrostatic columns with resulting greater pressure changes when accelerated); hence, their survival might be limited by physical factors and the ability of the various organ systems (primarily cardiovascular) to adapt to them.

Most of the birds that died during centrifugation were examined by Dr. Livio Raggi, Avian Pathologist in the School of Veterinary Medicine. No evidence of infectious disease was found. Although some pathological changes were noted (which will be reported elsewhere), no consistent syndrome could be established for acceleration death. However, it seems likely, in view of the complex relationship between initial size and survival and its interpretation, that several distinctly different pathologies may perhaps be involved.

It is evident from the data in Table 1 that the lethality in birds from chronic acceleration stress is not systematically different between the two sexes. When chicks are exposed to other environmental stresses, mortality is much more frequent in males than in females; hyperthermal stress (Wilson and Plaister, 1951), hypothermal stress in featherless mutants (Abbott, 1958), and hypoxic stress (Smith, unpublished). Selye (1950, p. 81) attributed this frequently observed difference in mortality to the influence of sex hormones on adrenocortical function: females generally have larger adrenal glands, and folliculoids enhance, whereas testoids depress, adrenal development. The failure of acceleration-stressed birds to conform to this general observation suggests a basic difference between this and other stresses for such animals. However, in some other species (rats, rabbits and cats, Britton et al., 1946) males were found to be more sus-

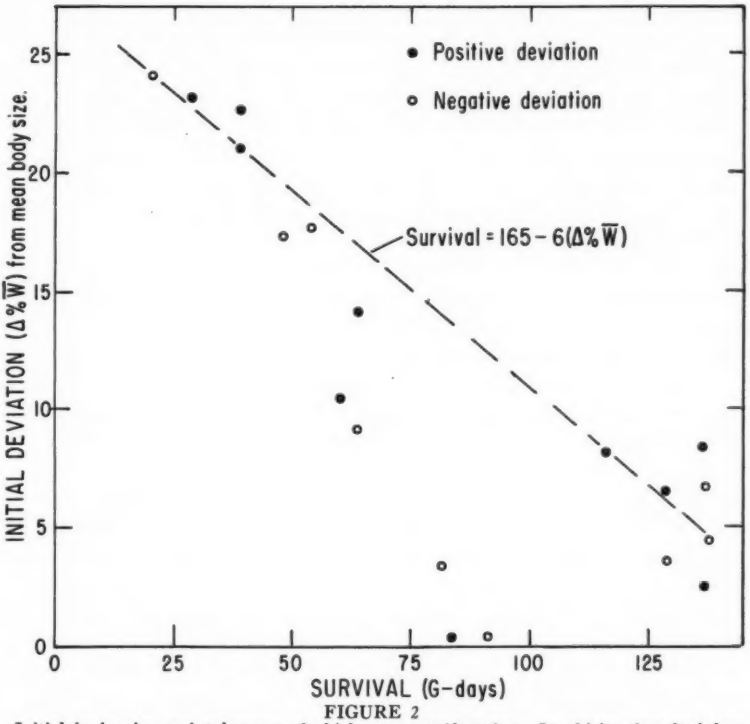

FIGURE 2

Initial body size and tolerance of chicks to centrifugation. In chicks that had been brooded normally before acceleration, survival during centrifugation was correlated with degree of conformity of body size to the mean of the group.

ceptible than females to acute and extreme acceleration stress; in rats castration and sex hormone treatment did not alter this difference in resistance.

Species and strain differences are also evident among birds in survival under chronic acceleration stress, and not all of these differences are correlated with mature body size (Table I). A similar interspecific variation in resistance to acute acceleration has been noted in a wide variety of species (Britton et al., 1946).

The distribution of mortality during centrifugation varied considerably between trials, even between those with similar animals and

acceleration schedules and approximately equal total mortality (Fig. 1). In the more acute experiments (seven weeks' exposure and up to 4 G acceleration) mortality was more-or-less continuous, but was greatest late in the experiment, during the 3.5- or 4-G period. This continual and increasing mortality suggested that perhaps little or no adaptation took place in the centrifuged birds. That possibility was tested in one experiment (trial B) by adding 8 control chicks (4 ♂ and 4 ♀) to the centrifuge at the start of the second week at 3 G. All of these chicks died within 72 hours (three within 24 hours); during this week the mortality of the centrifuged group was about 10% and total mortality up to that time was about 37%. Consequently, it appears that physiological adaptation plays some part in survival (rather than mere selection of susceptible individuals).

If the intensity of acceleration was moderate (2.5 G) but the exposure prolonged (24 weeks), mortality occurred in two distinct periods: early, during the first five weeks; and later, during the 14th to 19th weeks. According to Selye's (1950) general description of stress and adaptation, the early deaths may indicate birds that failed to adapt, and the later deaths, birds whose adaptive capacity was exhausted.

In all cases, growth was repressed by exposure to accelerative forces (Fig. 3). This does not reflect a deficiency of the centrifuge cages, since in the preliminary periods (without acceleration) the experimental birds grew more rapidly than their controls. The growth repression also does not appear to result from an immobilization of the experimental birds, representing merely an involuntary reduction of food intake. When acceleration was moderate (2.5 G or less) and direct observation of the centrifuging birds was possible, movement appeared reasonably free. At higher accelerations (3-3.5 G) considerable feed was consumed (though it could not be measured quantitatively with our apparatus), indicating that necessary movement could be accomplished during centrifugation. At the most intense acceleration employed (4 G), however, movement during centrifugation probably became quite difficult.

In experiments involving prolonged exposure (24 weeks) to moderate acceleration (2.5 G), growth repression appeared to take place in stages (Fig. 3). After two or three weeks of centrifugation the birds appeared to adapt to their new environment, and their growth rate again became comparable to that of the controls. However, as

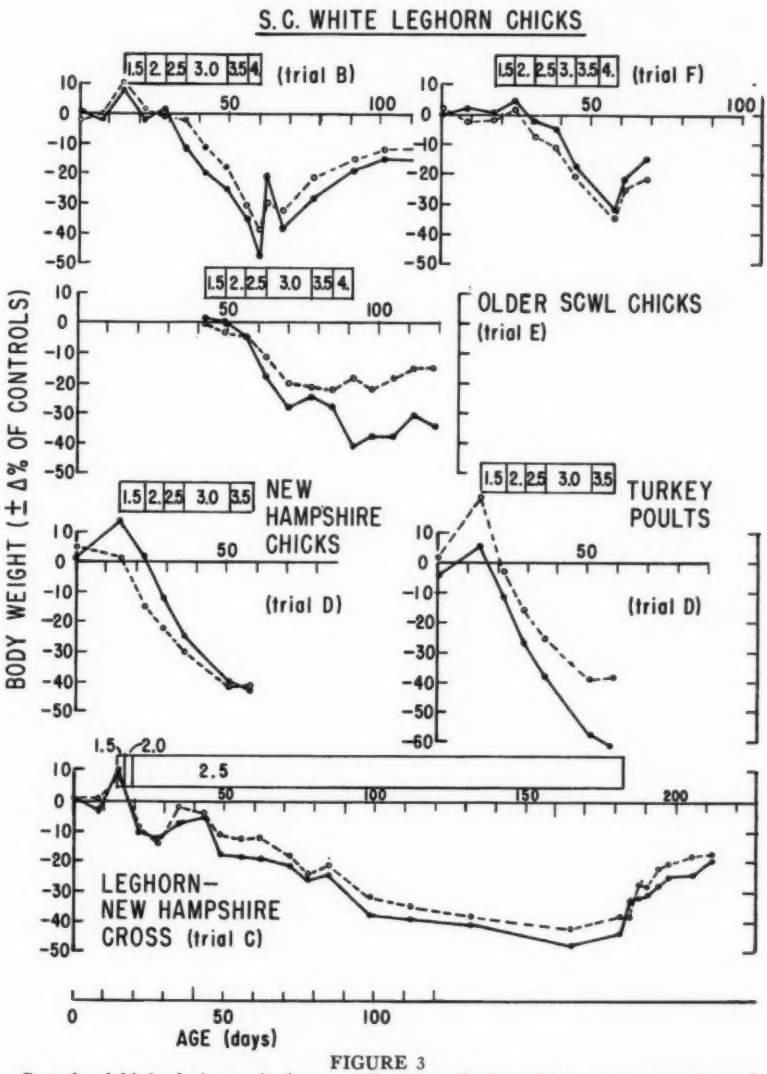
S.C. WHITE LEGHORN CHICKS
1.5 2. 2.5 3.0 3.5 4. (trial B)
1.5 2. 2.5 3. 3.5 4. (trial F)
1.5 2. 2.5 3.0 3.5 4.
OLDER SCWL CHICKS
(trial E)
1.5 2. 2.5 3.0 3.5
NEW HAMPSHIRE CHICKS
(trial D)
1.5 2. 2.5 3.0 3.5
TURKEY POULTS
(trial D)
1.5 2.0 2.5
LEGHORN–NEW HAMPSHIRE CROSS (trial C)
BODY WEIGHT (± Δ% OF CONTROLS)
AGE (days)

FIGURE 3

Growth of birds during and after centrifugation. Body weights of the experimental animals are presented as a percentage difference from their controls ($\Delta\% = \frac{x-c}{c} \times 100$). The acceleration schedule is indicated for each trial.

body size exceeded a particular limit, this adaptation no longer was protective, and another period of growth repression ensued. This sequence, which was repeated three or four times during the experiment, emphasizes the previously noted importance of body size to survival during chronic acceleration.

In trials involving shorter (7 weeks) but more intense acceleration (up to 4 G), growth depression was progressive, and at the end of the experiement the body mass of the centrifuged birds was only 40-50% of that of their controls.

Sexual maturity was also delayed by centrifugation. Birds exposed to 2.5 G for 24 weeks matured sexually and laid eggs while in the centrifuge, but 37 days later than did their controls (i.e., 177 days vs. 140 days). Shorter and more intensive exposure also delayed attainment of sexual maturity. However, once egg production started, no other differences were evident (Table II). This repressive effect of

TABLE II
EFFECT OF CENTRIFUGATION ON FEMALE REPRODUCTIVE FUNCTION

Trial B	Centrifuged	Control
Age at onset of lay	171 ± 3 days*	156 ± 4 days
Laying rate (215-245 days)	75.5 ± 2.6%	72.6 ± 3.9%
Egg size (230-245 days)	52.8 ± 1.3 gms.	53.5 ± 1.1 gms.

* Random probability of difference from controls <5%.

centrifugation on sexual development may be a general phenomenon; Britton et al., (1946) noted an inhibition of normal estrual activity in rats after exposure to intense acceleration.

Following centrifugation, the experimental birds underwent a period of rapid growth for about 40 days (Fig. 3). However, at the end of this time they were, and remained, about 10-20% smaller than were their controls. This is somewhat different from the response of chronically fasted rats upon refeeding (Jackson, 1936): females attained normal size, though males remained somewhat smaller. The return to normal gravity was not accompanied by any mortality.

DISCUSSION

The detrimental effect of environmental extremes and the adaptation of organisms to such conditions are well described, but as yet poorly explained (Selye, 1950; Adolph, 1956). Most of the physical factors in the environment are variable to some degree, and it is pos-

sible that the ability to adapt to extremes may have developed through natural selection during periodic exposure to extremes. The most constant of all physical factors in the environment is its accelerative force, gravity. Of course, acceleration and decelerations may result from various movements, but under natural conditions these can be only very brief. Prolonged centrifugation results in an added accelerative force, and is physiologically equivalent to a change in gravity. Since this sort of change is so unusual, the ability of organisms to adapt to it is of some interest.

The types of physiological changes induced by accelerative forces are fairly well known from short-term centrifugation studies, usually on man. The principal effect of centrifugation is an increase in weight (the product of the mass of a restrained body and the accelerative force to which it is exposed). Increased body weight increases the work involved in normal locomotion, etc. The densities (weight per unit volume) of body fluids are also increased during centrifugation; consequently, greater work is required for normal function of such organs as the heart, intestines, etc. Apparently most birds can withstand or adjust to changes induced by accelerative forces up to 2.5 G; although birds are severely stunted (i.e., reduced in body mass) by chronic acceleration, they appear to be normal, at least to the extent that they attain sexual maturity. The effects induced by more intense accelerative forces exceed the birds' ability to compensate or adapt, and very few birds can survive exposure for more than a few days to forces on the order of 4 G.

The period following removal from the centrifuge may be of special significance. Assuming that the birds had so adapted to acceleration that they became more-or-less normal, removal to normal gravity would be quantitatively equivalent to a sub-gravity situation. The ability of such birds to grow rapidly without significant mortality would appear to indicate that a minimum of physiological stress would accompany exposure to sub-gravity.

Summary

Exposing domestic birds to acceleration of 1.5-4 G for seven weeks was lethal to 70-90%, and the growth of survivors was repressed 40-60%. A lesser acceleration (2.5 G) for 24 weeks led to a similar growth repression (about 50%), but a lesser mortality (36%).

After centrifugation the experimental birds gained weight rapidly, eventually weighing only 10-20% less than their controls. No mortality was encountered in the post-centrifugation period. Exposure to accelerative forces delayed reproductive maturity significantly.

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EXPERIMENTAL STUDIES ON MITOMYCIN C* I.

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In 1956, Sugawara and Hata (25) reported the isolation of new antibiotics, called mitomycins, from *Streptomyces caespitosus*, sp. nov. Hata *et al.* (3, 4) demonstrated that the mitomycins possessed a strong activity against gram positive and gram negative bacteria, as well as against Ehrlich ascites tumor cells. Wakaki *et al.* (36, 37) in the course of cultivating the mold, found that besides the two fractions, Mitomycin A and B, a third fraction of a bluish-violet color was abundantly present in the culture. They called this antibiotic Mitomycin X which name was later changed to Mitomycin C. It is thermostable, with a high melting point and soluble in many organic solvents. Marumo *et al.* (8) reported that the culture of this mold contained some other active mitomycins.

A number of investigators confirmed the antitumor activity of Mitomycin C. Sugiura and Stock (26) reported that the dose 2 mg/kg. wt./day, given for 7 consecutive days had a completely destructive effect on a number of ascites and solid tumors, but did not inhibit Sarcoma T 241, Sarcoma MA 387 and some other tumors. Mitomycin C inhibited the Friend virus leukemia according to Sugiura and Stock (27). Sugiura (28) reported that oral administration of Mitomycin C, 10 mg./kg. wt./day for seven days caused a complete regression of Sarcoma 180 ascites tumor, Friend virus leukemia and carcinoma 1025. The growth of methylen-cholanthrene-induced sarcomas in mice was only partially inhibited by the antibiotic. Sugiura (29) also reported that a large part of mouse, rat, and hamster tumors re-

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sponded to the Mitomycin C therapy. Usubuchi (34) and Usubuchi *et al.* (35) found that Mitomycin C inhibited hepatoma 7974 but had only a slight inhibitory effect on Hirosaki sarcoma. This last observation was confirmed by Ohoshi (9). Various ascites and solid tumors in animals responded to the Mitomycin C therapy according to Reilly *et al.* (11); Sakai and Terenaka (9); Sakai *et al.* (13); Shiba *et al.* (16); Shima *et al.* (18); Shimada *et al.* (19); Sukie *et al.* (30); Terenaka (31, 32, 33); Usubuchi (34) and Usubuchi *et al.* (35).

Very litle information on the cytotoxicity of Mitomycin C in animals has been published so far, except the observations by several investigators that leukopenia was induced by the antibiotic. Kanamori *et al.* (6) revealed considerable shrinkage of the spleen of animals treated with Mitomycin C, was present. Sternberg *et al.* (24) reported that Mitomycin C destroyed hematopoietic tissue, injured intestinal epithelium, and caused hemorrhagic diathesis in mice, rats, and dogs, and was responsible for necrotizing nephrosis in Rhesus monkeys. The fate of Mitomycin C in the body of dogs was investigated by Schwartz (15).

While the experiments with Mitomycin C on animal malignant tumors demonstrated a remarkable activity of Mitomycin C, the clinical investigations with it were not very encouraging, so far. Shimada *et al.* (19) and Sukie *et al.* (30) reported beneficial effects in some patients treated with Mitomycin C. According to Shiraha *et al.* (20) in eighteen out of one hundred and one cases treated with Mitomycin C, there was a complete regression of the tumors. The side effects of the therapy were minimal. Leukopenia, present in almost all cases, was of a temporary nature. Shiba (17) stated that the Mitomycin C therapy had only slightly salutary effects on the cases he treated, and Colsky *et al.* (2), on the basis of their clinical trials, in the course of which 39 children and 85 adults received Mitomycin C, a total dose of from 0.75 to 1.5 mg./kilo wt., arrived at the conclusion that "objective responses have been minimal." They emphasized the high degree of Mitomycin C toxicity, which was manifested by leukopenia, hemorrhage, decrease in number of platelets and neutrophils, anorexia, diarrhea and occasional alopecia.

In the present study, various aspects of the Mitomycin C therapy on animals and fowls were investigated, with an emphasis on its cytotoxicity.

Material and Technique

Mitomycin C, in a pure form, was generously supplied to us by the Merck Institute for Therapeutic Research. We would like to express our gratitude to Dr. George E. Boxer of the Institute, and Dr. Max Tishler of the Merck, Sharp and Dohme Company.

Mitomycin C content of tissue. The content of Mitomycin C in the tissue of animals was estimated by the cylinder plate assays with *Bacillus subtilis*. A standard curve of Mitomycin C was prepared by diluting the standard solution to the concentrations from 1.5 to 0.6 gammas. Three plates were used for determination of each point on the curve. On each of three plates containing spores of *B. subtilis*, three cylinders with the 1.0 unit per milliliter standard solution and three other cylinders with the concentration under test, were placed. After the plates were incubated for 18 hours, the activity of a unit was calculated on the basis that the square of the diameter of the inhibition is proportional to the logarithm of the Mitomycin C concentration.

The tissue, spleen or liver, were removed from the animal under aseptic conditions, weighed and immediately frozen in an acetone-dry ice bath, and stored in the deep freeze. The tissue was homogenated, extracted with chloroform, filtered through a sterile sintered glass filter, and the filtrate evaporated in a vacuum desiccator at room temperature. Dried tissue was dissolved in 2 ml. of sterile distilled water, and 0.2 ml. of the solution was placed in each of 3 cylinders, on the three plates.

The data obtained with this assay was verified on some specimens by the ultraviolet measuring as described by Kagawa *et al.* (4).

Estimation of DNA and PNA in tumor tissue. Rats, bearers of a four-day transplant of Crocker carcinoma, were given Mitomycin C, 2 mg./kilo wt./rat/day, for four consecutive days, intravenously. Two hours after the last injection, the tumor was removed. They averaged about 1.5 cm. in diameter. A modified method of Barnum *et al.* (1) and Payne *et al.* (10) as described by us in our previous publications (21, 22) was used for the extraction and separation of DNA and PNA. The technique of Schneider (14) was employed for estimation of DNA and PNA in the tumor tissue. The estimation of the nuclear DNA was made according to the procedure recommended by Lessler (7) as described previously by us. The DNA content per cell, in arbitrary units was calculated accordingly.

Glycolysis in tumor tissue. The effect of Mitomycin C on anaerobic glycolysis in tumor tissue was investigated by Warburg's procedure (37). Fresh Crocker rat carcinoma was sliced to a thickness of 0.6 mm., and the slices were suspended in an isotonic mixture of Ringer's solution and sodium bicarbonate in a Warburg apparatus. The same amount of fresh tissue was used in each flask. The rate of anaerobic glucose metabolism was measured by the CO_2 liberated from bicarbonate by lactic acid produced by the tumor cells.

Antiviral activity. The activity of Mitomycin C against viruses was investigated by the chick embryo technique. Mouse influenza virus PR8, 0.1 ml. saline was inoculated into the chick embryonic cavity, and fifteen-twenty minutes thereafter, Mitomycin C, in various concentrations, 0.1 ml. saline, was introduced into the same cavity. After a 24-hour incubation period, the titration, following the Salk pattern, was made. In the case of Rous sarcoma virus, the virus, density 10^{-1}, 0.1 ml., was inoculated onto the chorioallantoic membrane of a 10-day-old embryo, and twenty or twenty-five minutes thereafter, Mitomycin C, 0.1 ml., in various concentrations was inoculated also onto the membrane. The method of counting lesions and titration were essentially the same as described by us in our previous paper (23). This procedure was supplemented in some tests by the technique of intravenous inoculation of Rous virus, 10^{-1}, 0.1 ml. to a 12-day-old embryo. Shortly afterward, Mitomycin C, 0.1 ml., in various concentrations was inoculated intravenously, the embryos were killed at the intervals of 12, 24, 48 and 72 hours, and the slides examined histologically.

In the case of chick intravascular lymphomatosis, Beard's virus, Strain "R," was inoculated intravenously, Log 4, in 0.2 ml. Simms solution, to ten-day-old chicks. Mitomycin C was administered subcutaneously.

Antitumor activity. Two types of ascites tumors, Ehrlich carcinoma and mouse sarcoma 180 served us for testing the antitumor activity of Mitomycin C. The original inoculum 0.2 ml. saline contained from 1.5 to 4.0 million ascites tumor cells, injected intraperitoneally. The cell counts were taken at the intervals of every two or three days. The Mitomycin C therapy was initiated at various times after cell inoculation, depending on the type of experiment. For histological investigation of the adrenals, Sudan III was used, and hematoxylin-eosin, Feulgen and other stains for various tissues.

Experimental

Assays on Mitomycin C Toxicity

Consecutive Application. Twenty young albino rats, all males, thirty-three days of age, were divided into two groups. One group served as control; the second group of ten rats, received 2.5 mg./kilo wt./day/rat for six consecutive days, or a total dose 15.0 mg./kilo wt. of Mitomycin C. The antibiotic was administered subcutaneously. The acute toxic effect was apparent after four days of treatment. One rat died on the fifth, one on the sixth, and one on the seventh day after the treatment started. The rest survived, but were in poor general condition, showing hemorrhagic diathesis, which persisted for a week or so. Leukopenia was present in all treated rats but in various degrees of gravity. The number of which blood cells varied from 1,900 up to 3,483, with the control rats having an average wbc of 5,090. The reduction of weight of the spleen was greater proportionally than that of their weight. Table I summarizes the results of this assay.

TABLE I

Effect of Mitomycin C on Young Rats

2.5 mg./kilo wt./day/rat for six consecutive days, subcutaneously 10 control and 10 treated rats

Average figures at the end of assay

Groups	Gm. Weight, days 1	4	7	WBC after 7 days	Liver mg. after 7 days	Spleen mg. after 7 days
Control	32.82	44.76	52.2	5090	2220	190
Treated*	33.75	36.54	31.98	2463	1350	86

* Three rats died during the seven day period.

When in another series of tests with rats, average weight 180 gm., the dose of Mitomycin C was reduced to 2 mg./kilo wt./day/rat for six consecutive days, the toxic effect of this drug was somewhat less pronounced but the side effects followed the same pattern as in the previous assays.

The baby chicks showed a slightly higher resistance to Mitomycin C toxicity. Twenty baby chicks, six-days of age, white Leghorn, were divided into two groups. One—control; second group received 19 mg./kilo wt./chick during sixteen day period. The mortality rate in the treated group was 50 per cent. Leukopenia was moderate by the end of the experiment. There were present mild neutropenia and

lymphocytosis. The growth of chicks was retarded by about 40%, as was the weight of the liver and heart reduced. The spleen, however, was about three times smaller in the treated group than in the control. The results are presented in Table II.

TABLE II
EFFECT OF MITOMYCIN C ON BABY CHICKS
19 mg./kilo wt./chick during 16 days. Consecutive application, subcutaneously.
10 control and 10 treated.
Average figures at the end of assay.

Groups	Weight gm.	Weight mg. Liver	Heart	Spleen	WBC	Lympho %	Neutro %	Mono %
Control	175.8	5955	1218	405	6310	67.4	18.7	8.1
Treated*	101.4	3650	780	120	3316	80.1	15.2	4.7

* Five chicks died.

Administration of Mitomycin C for a longer period, with a total dose of 23 mg./kilo wt. for twenty-two days, resulted in further shrinkage of the spleen. In some chicks the weight of the spleen was reduced to 80-100 mg. as compared with 800-920 mg. for control. Photo 1 gives the comparative size of the chick spleens: the Mitomycin C treated, and the control.

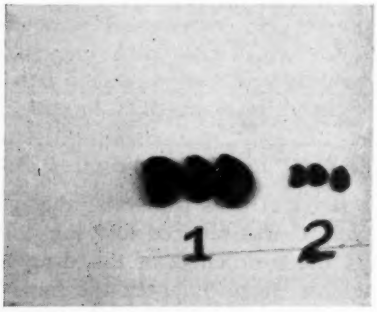


PHOTO 1.
1. Control chicks, spleen, average weight, 889 mg.
2. Chicks, received the total dose, 23 mg./kilo wt/chick, Mitomycin C. Spleen, average weight 95.4 mg.

Intermittent Application. A total dose, 20 mg./kilo wt. was administered to ten chicks, 4 days of age, and ten rats, 60 days of age. The antibiotic was given twice a week, for five weeks. Ten chicks and ten

rats, of the same age, served as control. Table III gives the average figures of this assay.

TABLE III

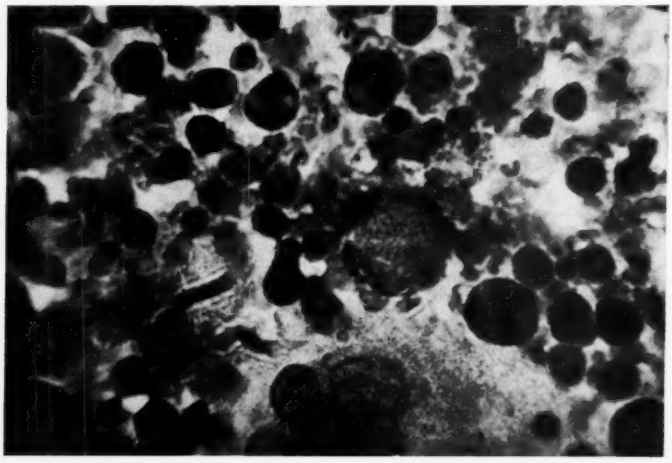
EFFECT OF MITOMYCIN C ON RATS AND CHICKS, INTERMITTENT APPLICATION
A total dose 20 mg./kilo wt.
Injected subcutaneously twice a week, for five weeks.
Chicks: 10 control, 10 treated.
Rats: 10 control, 10 treated.
Average figures

Groups	Weight					Liver, mg. after 33 days	Spleen, mg. after 33 days
	0	6	18	25	33		
Chicks							
Control	50.7	73.7	173.6	288.9	387.6	7210	768
Treated*	50.6	77.2	163.8	236.5	304.1	5965	318
Rats							
Control	80.7	115.8	135.8	155.6	171.4	9123	495
Treated*	88.1	100.1	117.7	129.4	137.3	7346	234

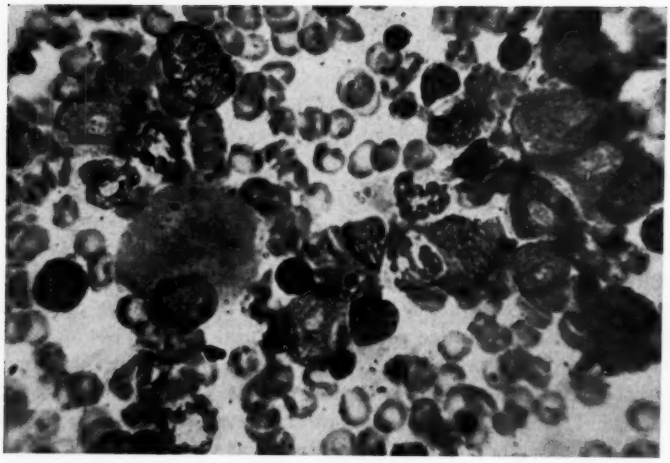
* There was no mortality in the treated groups.

Both rats and chicks, receiving Mitomycin C intermittently, were in a satisfactory condition, with moderate leukopenia present, and no mortality among them was recorded. Although there was a retardation in chick and rats growth, it was in the limit of 20-23 per cent. The injury to the spleen was apparently less pronounced.

The effect of Mitomycin C on the blood of chicks and rats was not uniform. Some baby chicks, after having received the total dose 20 mg./kilo wt./chick, administered consecutively, showed considerable leukopenia. Their white blood count went from an average 5,000 down to 1500-1800. On the other hand, there were chicks, of the same age, who apparently tolerated Mitomycin C much better, and the number of white blood cells was only slightly decreased, often about 3,500 or even more. Sternberg *et al.* (24) reported that this antibiotic destroyed the hematopoietic tissue. We are unable to confirm this observation on rats or mice receiving the total dose of 14 mg./kilo wt., a dose which arrested almost completely the ascites tumor cells growth. Photo 2 shows the bone marrow of a rat which was given a total dose, 15 mg./kilo wt. on seven consecutive days. No pathologic changes are detected. The bone marrows are quite cellular. Myeloid cells, erythroid cells and megakaryocytes appear to be present in normal quantity. There is no sign of aplastic process.



A.



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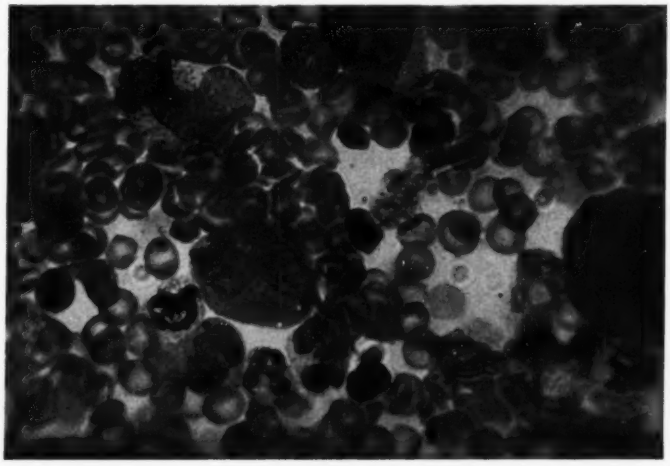


C.

PHOTO 2.

White rats, receiving the total dose, 15 mg./kilo wt./rat, for seven consecutive days. Bone marrow. Wright, x 970. Oil imm.

A. Rat # 103.
B. Rat # 104.
C. Rat # 105.

Platelets appeared to be present in approximately normal amounts, and there was no indication that the number of platelets in rats or mice was decreased to any considerable degree. Again, there was some variation. In some rats, the number of platelets was 650,000, but in others, it remained as high as 1,000,000, with the control rats, of the same age, showing an average 900,000 platelets.

Colsky *et al.* (2) reported a decrease in neutrophils in humans receiving Mitomycin C therapy. In animals, rats, mice, and baby chicks, there was a very moderate trend toward neutropenia, and lymphocytosis. Control rats had an average: 28 per cent neutrophils, 4% monocytes, 63% lymphocytes and 1% eosinophils. After four days of Mitomycin C therapy, 2 mg./kilo wt./day/rat, the neutrophils went down to an average 21%, monocytes 5%, eosinophils 1% and lymphocytes 73%. After seven days therapy: neutrophils 17%, monocytes 2%, eosinophils 2%, lymphocytes 79%.

The liver and spleen of rats, receiving the total dose 14 mg./kilo wt./rat showed very little changes. Proliferated erythrocytes and erythroblasts were observed in the hepatic parenchyma, and some Kupffer cells appeared to be swollen and vacuolized. The splenic lymphocytes were slightly decreased in number, with lymphonodulous linis slightly enlarged. The spleen of baby chicks, which were given a total dose of 24 mg./kilo wt., showed considerable changes. The white pulp had disappeared and the lymphonoduli were not distinct. The lymphatic cells were not detectable and the production of lymphocytes was not visible. Numerous immature erythrocytes infiltrated the trabeculae. The adrenals of chicks showed a moderate decrease in lipids of the cortical cells when stained with Sudan III with the spongiocytes vacuolized and somewhat swollen.

Effect of Mitomycin C on Glycolysis. Crocker rat carcinoma was used for testing the effect of Mitomycin C on glycolysis in tumor tissue. Approximately the same amount of fresh tissue (about 25 mg. dryweight) was placed in three flasks. One flask, control, with a bicarbonate-Ringer's solution, containing 0.05 m. glucose, to two other flasks, 0.1 mg. and 0.02 mg. Mitomycin C, were added to the same solution with glucose. The rate of CO_2 evolution in all three flasks was recorded every eight minutes. There was no inhibition of glucose metabolism.

Effect of Mitomycin C on Nucleic Acids in Tumor Tissue

Total PNA and DNA content of rat carcinoma. Sixteen rats, males, average weight 150 gm. were divided into two groups: I. control and II. Mitomycin C treated, each of eight rats. Seventy-two hours after carcinoma G-175 was transplanted, the rats of Group II received intravenous injections of Mitomycin C, 2 mg./kilo wt./rat, for four consecutive days. The tumors of both groups were removed on the seventh day after transplantation. The total PNA and DNA of tumor tissue was estimated following Schneider's method (14). Table IV summarizes the results of these assays.

These tests indicate that Mitomycin C had no effect on the total PNA, but exerted an inhibiting influence upon the total DNA in the tumor tissue, under these conditions of experimentation.

Nuclear DNA content of rat carcinoma. Six rats, bearers of carcinoma G-175, a 72 hour transplant, received the intravenous inocula-

TABLE IV
EFFECT OF MITOMYCIN C ON THE PNA AND DNA CONTENT OF RAT CARCINOMA G-175
Total dose of Mitomycin C injected intravenously,
2.0 mg./kilo wt./day/rat for four days.

Groups	Tumors	PNA per cent of dry wt.	DNA per cent of dry wt.	PNA/DNA ratio
Group I				
Control	7	2.43 ± 0.18	3.05 ± 0.18	0.79
Group II				
Mitomycin C	8	2.46 ± 0.11	2.23 ± 0.21	1.1

tion of Mitomycin C, 2 mg./kilo wt./day/rat, for four consecutive days. Two hours after the last injection of Mitomycin C, the tumors were removed and the nuclear DNA content of the tissue was estimated with the Feulgen reaction. Six rats, of the same average weight, and bearers of the carcinoma transplanted at the same time, served as control. The tumors in the treated group, were an average 1.6 cm. in diameter. The control tumors were 2.2 cm. in diameter. The results are presented in Table V.

TABLE V
EFFECT OF MITOMYCIN C ON THE NUCLEAR DNA IN RAT CARCINOMA G-175
Feulgen reaction.
Mitomycin C, a total dose 8 mg./kilo wt./rat administered intravenously
Average figures

Groups	No. of specimens*	No. of cells	(E) x (A)** Arbitrary units	
Group I				
Control	5	100	4.01	0.11
Group II				
Mitomycin C	6	125	3.34	0.08

* Each specimen taken from a different tumor.
** E = extinction coefficient.
A = nuclear area in D^2.

Mitomycin C, administered to rat carcinoma G-175, with a total dose of 8 mg./kilo wt./rat, decreased the nuclear DNA content of the tissue.

Mitomycin C content of liver and spleen. The Mitomycin C content of liver and spleen of the rats receiving Mitomycin C injections was estimated by the cylinder plate assays with *Bacillus subtilis,* which was described above. Rats, average weight 332 gm., receiving Mitomycin C, 1 mg./kilo wt./day/rat subcutaneously, for seven consecu-

tive days. Some rats were sacrificed after four, the others after six and seven daily injections, and their liver and spleen were removed. Table VI gives the results of the assays.

TABLE VI

MITOMYCIN C CONTENT OF LIVER AND SPLEEN OF RATS

Group I. total dose Mitomycin C, 4 mg/kilo wt./rat
Group II. total dose Mitomycin C, 6 mg/kilo wt./rat
Group III. total dose Mitomycin C, 7 mg/kilo wt./rat
administered consecutively

Groups	Av. Wt. Liver gm.	Mito. cont. total liver gamma	Mito. cont. liver per gm. tissue gamma	Av. Wt. Spleen gm.	Mito. cont. total spleen gamma	Mito. cont. spleen per gm. tissue gamma
Group I						
4 days, 1 mg/kilo wt/day/rat	11.138	8.9	0.8	1.215	5.0	4.0
Group II						
6 days, 1 mg/kilo wt/day/rat	11.188	13.0	1.17	1.305	6.0	4.61
Group III						
7 days, 1 mg/kilo wt/day/rat	12.235	14.9	1.21	0.916	7.7	8.4

There seems to be a definite accumulation of Mitomycin C in the spleen of rats, receiving this antibiotic, as calculated both totally and per gram of fresh tissue. An increase in the content of Mitomycin C, per gram of fresh tissue, was also noted in the liver, although in a lesser degree than in the spleen.

Effect of Mitomycin C on Rous Sarcoma Virus and Chick Lymphomatosis

As a matter of routine screening, Mitomycin C was tested on several viruses by the chick embryo technique. It was found that the antibiotic possessed a definite, yet moderate antiviral activity. Table VII gives the data on the effect of Mitomycin C on mouse influenza virus PR8.

The dose, 0.05 mg./0.1 ml./embryo was found to be quite toxic. The smaller doses, 0.03 and 0.04 mg. were less toxic and inhibited influenza virus at 10^{-5} and 10^{-6}.

TABLE VII
EFFECT OF MITOMYCIN C ON INFLUENZA VIRUS PR8
Chick embryo CA cavity
Virus inoculum: 0.1 ml.
Incubation: 24 hours
Titration: Salk's pattern

Groups	Control PR8	Mitomycin C 0.03 mg.	Mitomycin C 0.04 mg.	Mitomycin C 0.05 mg.
Number of eggs	50	50	50	50
ID50	7.7	6.5	6.1	5.9
Mortality rate	0	10%	24%	35%

Rous virus. Two methods of testing the activity of Mitomycin C on Rous virus were used: (A) the CA membrane technique and (B) the intravenous inoculations to chick embryo.

(A) The Rous sarcoma virus, obtained from the American Type Culture Collection, was diluted in Simms salt solution, to which 1% Bovine albumin (Armour Laboratories) was added. The virus, density 10^{-1}, 0.1 ml. was inoculated onto the chorioallantoic membrane of a ten-day-old chick embryo. Mitomycin C, 0.02, 0.03, 0.04 and 0.05 mg. in 0.1 ml. saline was inoculated 15-20 minutes after virus inoculation. The eggs were incubated for seven days and the number of lesions and their size were recorded. The results are summarized in Table VIII.

TABLE VIII
Chorioallantoic Membrane Method
Rous virus 10^{-1}, 0.1 ml.
Mitomycin C inoculated onto membrane 15 minutes thereafter. 10-day-old embryos.
Incubation 7 days.
Inhibition is estimated as percentage of the activity of the control which was taken to be one hundred per cent.
Average for two tests, each of 30 eggs.

Mitomycin C concentrations mg.:	0.02	0.03	0.04	0.05
Inhibition Percentage	12	18	23	28
Mortality Rate Percentage	5	12	28	60

The dose, 0.05 mg./0.1 ml./embryo of Mitomycin C was too toxic for any proper estimation of its inhibitory activity on Rous virus. The doses 0.02, 0.03 and 0.04 mg./embryo moderately inhibited Rous virus.

(B) The method of intravenous inoculations of Mitomycin C to

chick embryo gave us additional information about the inhibitory activity of the antibiotic on Rous virus. Rous virus 10^{-1}, 0.1 ml. saline, was inoculated intravenously to a 12-day-old embryo. Twenty minutes thereafter, Mitomycin C was inoculated, also intravenously, 0.03 and 0.04 mg./0.1 ml. saline. The embryos were killed at the intervals of 12, 24, 48, and 72 hours, and examined histologically.

Results: Control. Twelve hours after inoculation, the endothelial cells of the blood vessel wall were swollen, with red blood cells visible both in the endothelium and tunica intima. Twenty-four hours after inoculation, the intercellular hemorrhage extended to the tunicas media and adventitia, with many cells in the endothelium partially or completely destroyed. After forty-eight hours, extravascular small lesions are detected with hemorrhages in the vicinity of the capillaries. Small hemorrhages in the liver. Spleen normal. Seventy-two hours' examination revealed swollen hepatic and Kupffer cells, the vacuolization of the cells of hepatic lobuli and the appearance of spindle-shaped sarcomatous cells on the edge of the hepatic parenchyma.

Mitomycin C. The dose 0.05 mg./0.1 ml. Mitomycin C was too toxic, killing about sixty-five per cent of embryos by 48 hours. The mortality rate with the dose 0.04 mg. was about 10 per cent. The intercellular hemorrhage was present both in the endothelium and in the tunica intima twelve hours after inoculation. However, its expansion and other cellular changes in the tunicas media and adventitia were retarded and somewhat less pronounced when compared with the control. There were no extravascular lesions observed at the forty-eight hour examination. At 72 hours, the liver had hemorrhagic areas and a few small sarcomatous lesions.

Comment: The results of our investigation indicate that Mitomycin C, at a dose 0.04 mg. possessed a moderate inhibitory activity upon Rous sarcoma virus in both tests conducted by us: the chorioallantoic membrane method and the intravenous inoculations to chick embryo.

Chick intravascular lymphomatosis

Forty chicks, white Leghorn, ten-day-old, were inoculated intravenously with Beard's virus, strain "R," Log 4, 0.2 ml. Simms solution. They were divided into two groups each of 20 chicks. One group served as control. The second group received Mitomycin C, 2 mg./kilo wt./chick for seven consecutive days, subcutaneously. The results of these trials are summarized in Table IX.

TABLE IX
EFFECT OF MITOMYCIN C ON CHICK INTRAVASCULAR LYMPHOMATOSIS
Beard's virus strain "R", 0.2 ml., Log 4
Mitomycin C, 2 mg./kilo wt./chick/day for seven consecutive days
Chicks, ten-days-old, 20 control; 20 treated

Groups:	Mortality rate percentage	Average survival time days
Control	91	24.5
Treated	87	34.5

The Mitomycin C therapy, with a total dose 14 mg./kilo wt./chick, did not reduce in any significant degree the mortality rate of chicks infected with Beard's virus, strain "R," but influenced slightly the survival time of the treated chicks.

Effect of Mitomycin C on Ascites Tumor Cells

Consecutive Therapy. Two ascites tumors, Ehrlich mouse carcinoma and mouse sarcoma 180, were used in our experiments. The degree of antitumor activity of Mitomycin C on ascites tumor cells depends on several factors: the total dose of the antibiotic, the number of tumor cells initially inoculated, and the time table of its application. In our tests, for the sake of uniformity, Mitomycin C was injected intraperitoneally, in 0.1 ml. saline. Table X presents the results so obtained.

TABLE X
INFLUENCE OF THE NUMBER OF INITIAL INOCULUM TUMOR CELLS
Mitomycin C, 0.05 mg./mouse/day
Administered on seven consecutive days
Starting the day of cell inoculum

Days, Cell Count Average	Initial inoculum 1,500,000-2,000,000	2,500,000-3,800,000	4,400,000-5,000,000
Ehrlich Carcinoma			
Treated			
First day after inoculum:	5,600,000	12,300,000	18,350,000
Second:	15,340,000	25,425,000	37,436,000
Eighth:	32,000*	160,630	360,350
Tenth:	—	27,000**	235,450
Control			
First day after inoculum:	5,950,000	11,450,000	16,340,000
Second:	20,368,000	32,490,000	49,560,000
Eighth:	946,470,000	1,121,120,000	1,192,220,000
Tenth:	834,370,000	936,570,000	1,094,000,000

* 75 per cent of mice free from tumor cells.
** 60 per cent of mice free from tumor cells.

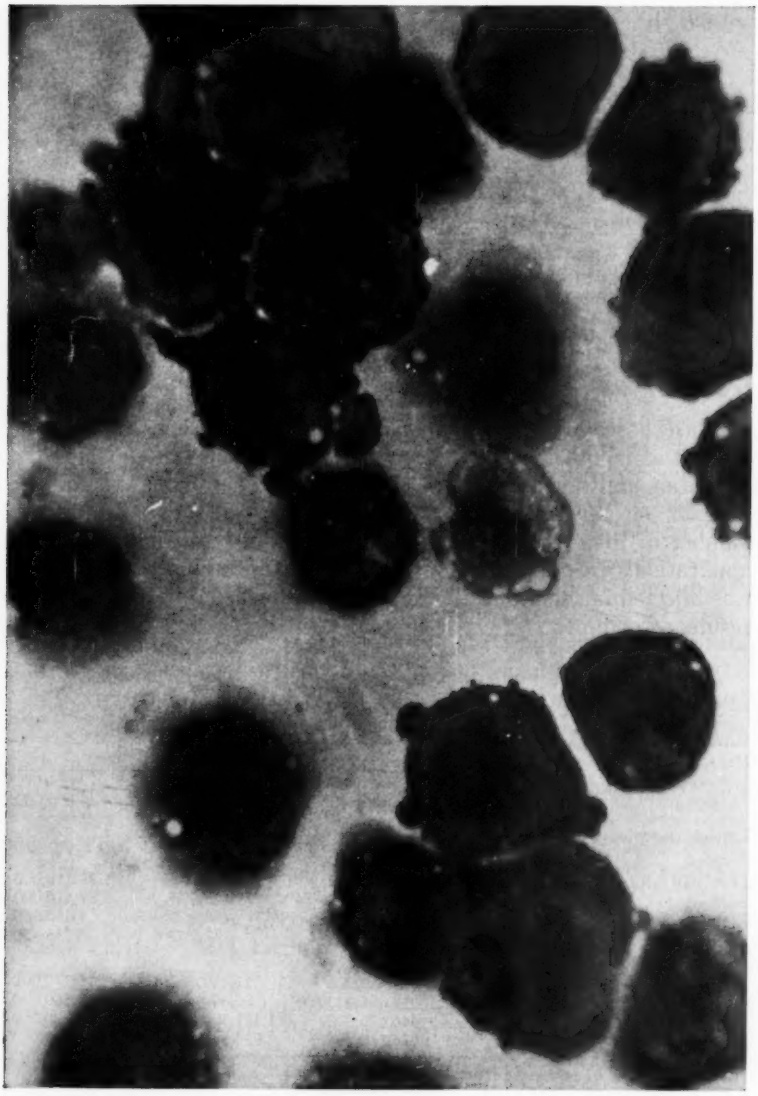


PHOTO 3.
Ehrlich carcinoma ascites cells. 24 hours after a single intraperitoneal injection of 0.04 mg. Mitomycin C. Giemsa, x 970. Oil imm.

The figures of Table X indicate that when the initial cell inoculum was as high as 4.4 or 5 million cells, Mitomycin C, the dose 0.5 mg./mouse/day for seven consecutive days, was unable to arrest the Ehrlich ascites cells multiplication. Similar results were obtained when the initial cell inoculum was 2 million, but the Mitomycin C therapy was started 48 or 72 hours after the inoculation. The total dose of Mitomycin C, 3.5 mg./mouse, did not arrest cell proliferation and by the ninth or tenth day, the number of ascites cells was above one million.

The dose of 0.02 and 0.03 mg./mouse/day administered for seven consecutive days, beginning the day of inoculum, produced only partial inhibition of Ehrlich and sarcoma 180 ascites tumors, unless the initial cell inoculum was only one million cells. The most effective dose was 0.05 mg./mouse/day for seven consecutive days, but the toxicity of this dose was apparent on the fourth and fifth day after the therapy was started.

The cytologic examination of ascites tumor cells of mice, receiving a single dose 0.04 mg. Mitomycin C, revealed certain interesting features of its activity. Twenty-four hours after intraperitoneal injection of Mitomycin C, the ascites cells showed very little, if any, significant changes (Photo 3). After forty-eight hours, a few small vacuoles appeared in the cytoplasma (Photo 4).

After seventy-two hours, some cells are partially destroyed, with many vacuoles visible both in the cytoplasm and the nucleus, while some tumor cells remained intact (Photo 5).

Ninety-six hours after, many cells are in a state of disintegration, the cytoplasma is grossly vacuolized, yet some nuclei retain an almost normal appearance (Photo 6).

Intermittent Therapy. The intermittent therapy of Mitomycin C, applied to ascites tumors, seems to have some definite advantages over the consecutive treatment. The toxic side effects of the therapy were less pronounced and the antitumor activity remained unchanged. In this series of experiments, five groups of mice, averaging 20.8 gm., were used.

Group I. Control, receiving no Mitomycin C.

Group II. Mitomycin C, 0.04 mg./mouse/day for seven consecutive days.

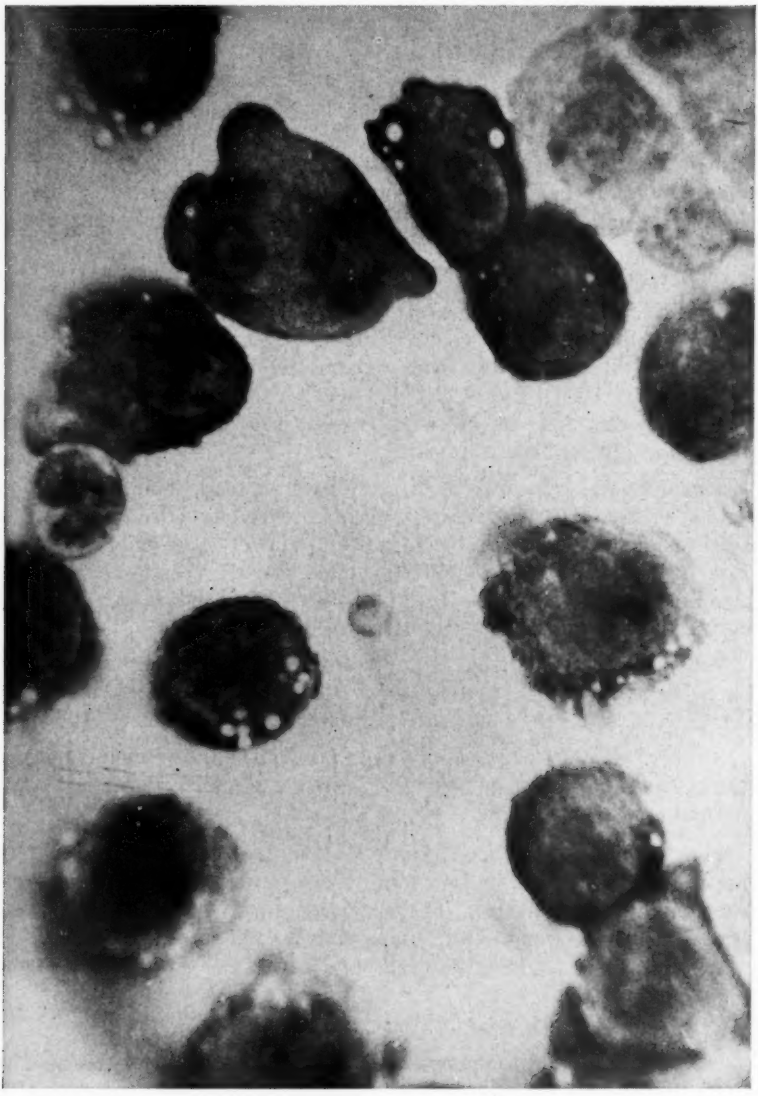


PHOTO 4.
Ehrlich carcinoma ascites cells. 48 hours after a single intraperitoneal injection of 0.04 mg. Mitomycin C. Giemsa, x 970. Oil imm.

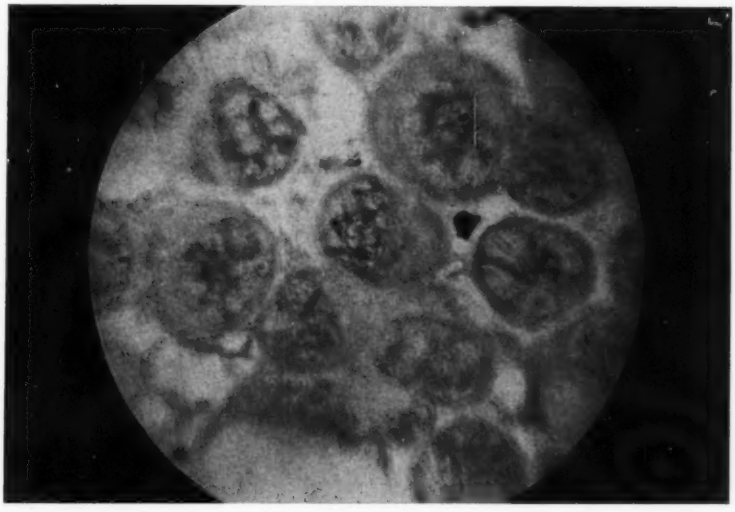


PHOTO 5.
Ehrlich ascites tumor cells. 72 hours after a single injection of Mitomycin C, 0.05 mg. Feulgen reaction. x 970.

Group III. Mitomycin C, 0.05 mg./mouse/day for seven consecutive days.

Group IV. Mitomycin C, 0.04 mg./mouse/day intermittently injected on the 1, 2, 5, 6, 9, 10 and 13th days.

Group V. Mitomycin C, 0.5 mg./mouse/day intermittently injected on the 1, 2, 6, 8, 12, 13, and 16th days.

The mortality rate for these five groups was:

Group I. Control, 100% dead.
Group II. 0.04 mg. consecutive, 90% died from cancer.
Group III. 0.05 mg. consecutive, 60% died from cancer.
Group IV. 0.04 mg. intermittent, 20% died from cancer.
Group V. 0.05 mg. intermittent, zero dead, all survived.

From all our experiments with Mitomycin C, administered to mice, bearers of ascites tumors, with various doses and times of applications, the results with the intermittent therapy, as obtained in Group V, were the most encouraging. As the best illustration of a decrease in

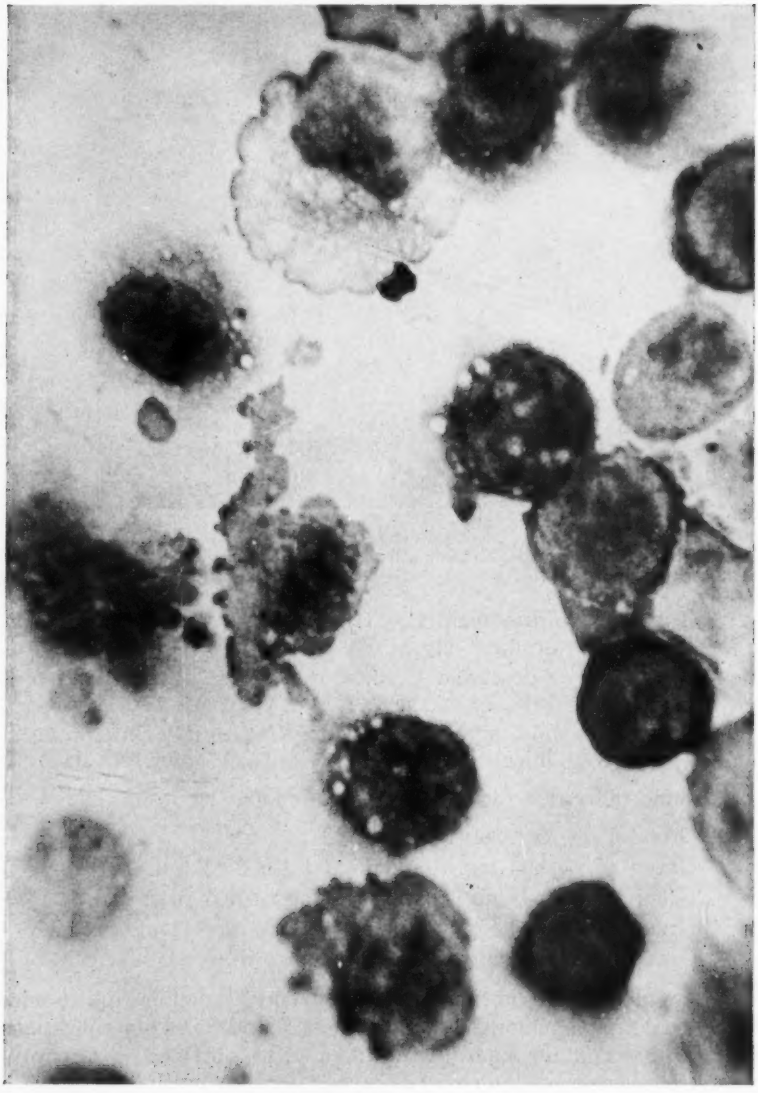


PHOTO 5A.
Ehrlich carcinoma ascites cells. 72 hours after a single intraperitoneal injection of 0.04 mg. Mitomycin C. Giemsa, x 970. Oil imm.

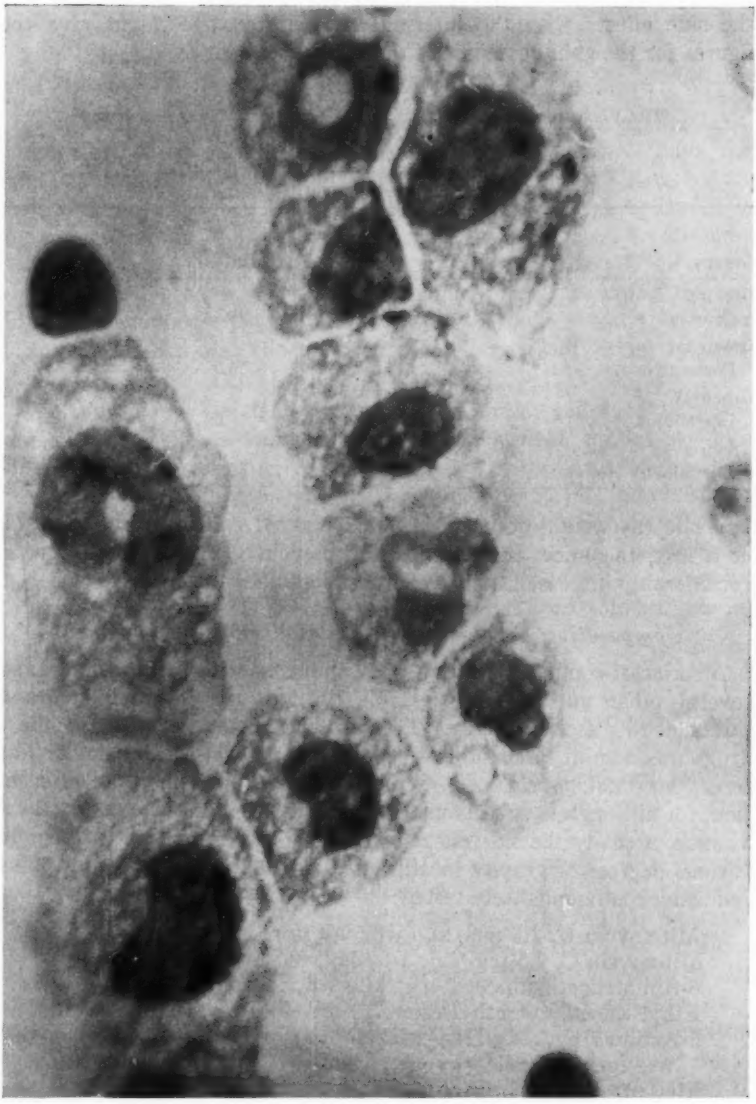


PHOTO 6.
Ehrlich carcinoma ascites cells. 96 hours after a single intraperitoneal injection of 0.04 mg. Mitomycin C. Giemsa, x 970. Oil imm.

the side effects with the intermittent therapy, one might give the figures for the gain in weight of the mice which survived.

TABLE XI

THE WEIGHT OF MICE TREATED WITH MITOMYCIN C AND SURVIVED

Group II, 0.04 mg. consecutive, seven days
Group III, 0.05 mg. consecutive, seven days
Group IV, 0.04 mg. intermittent, seven injections
Group V, 0.05 mg. intermittent, seven injections

Groups	Original wt. avg. gm.	Weight 25 days after, avg. gm.	Wt. gain avg. gm.
Group II			
Consecutive	22.72	21.5	Loss 1.22
Group III			
Consecutive	22.34	23.0	Gain .64
Group IV			
Intermittent	23.85	29.0	6.15
Group V			
Intermittent	24.61	30.2	5.51

While the weight gain of the mice, treated consecutively, was nil, or a loss, the mice, treated with Mitomycin C intermittently, have considerably increased their weight.

Comparative Antitumor Activity of Various Compounds

A series of comparative tests was conducted with Mitomycin C and several other antitumor drugs, on Ehrlich ascites carcinoma and sarcoma 180 ascites tumor. The maximal non-toxic dose of these drugs was administered subcutaneously for six consecutive days. The term "maximal non-toxic dose" should be taken with some reservation, for although it was claimed by the investigators who tested them as such, actually the side effects caused by the drugs were present in various degrees of gravity in all our mice receiving these drugs. The following compounds were tested:

Mitomycin C, 2.5 mg./kilo wt./day, for seven days.
Mitomycin C, 2.5 mg./kilo wt./day, for six days.
6-Aminonicotinamide, 2 mg./kilo wt., for eight days.
Ethyl thiosulfinic ethyl ester, 30 mg./kilo wt., for six days.
5-Amino-7-hydroxy-IH-v-triazolo (d) pyrimidine, 150 mg./kilo wt., for six days.
Methotrexate, 1.5 mg./kilo wt., for six days.

6-Mercaptopurine, 40 mg./kilo wt., for six days.
6-Thioguanine, 2.5 mg./kilo wt., for six days.
Methioprim, 300 mg./kilo wt., for six days.

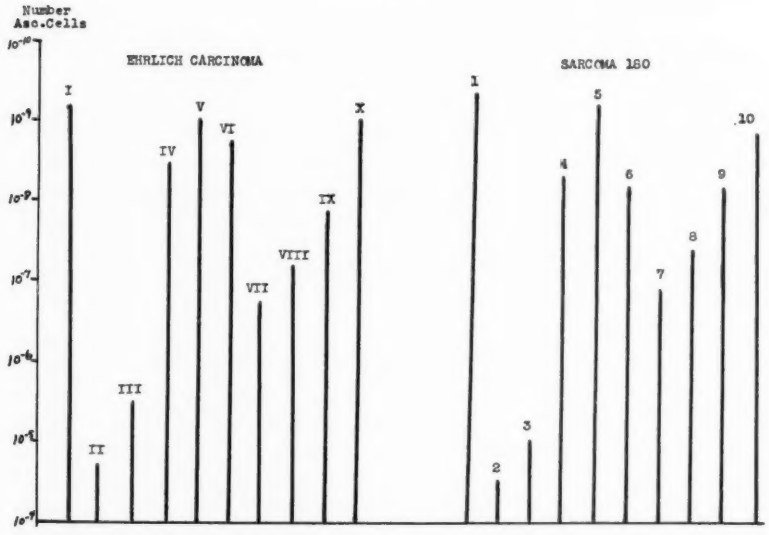
In all tests, the initial inoculum was about 2,000,000 ascites cells, and the cell count was made at the interval of two or three days, the final count on the eighth day after initial cell inoculum. Chart I gives the number of ascites cells on this final count on the eighth day. The antitumor activity of Mitomycin C seems to be superior to all these tested drugs, according to the figures presented in Chart I. Groups I and 1, were the controls, with the number of cells reaching the one billion cell number. Groups II and III, and Groups 2 and 3, were for Mitomycin C, 2.5 mg./kilo wt./mouse/day for six and seven consecutive days.

We may stress the fact that when the initial cell inoculum was smaller, about one million cells, and the treatment continued for a longer period, the destructive action of some of the tested compounds was much more pronounced. Thus 6-Mercaptopurine, administered for seven days, 40 mg./kilo wt. to mice bearers of Sarcoma 180 or Ehrlich carcinoma ascites tumors, with an initial cell inoculum of 1,000,000, destroyed the tumor cells almost completely. The same we found in regard to 6-Thioguanine, with a dose 3.0 mg./kilo wt. administered for seven days.

Discussion

All antitumor drugs, so far discovered, are toxic. Mitomycin C is not an exception to this general rule. It appears as if the toxicity of an antitumor drug is a part, or an attribute of its biological activity. At a certain point of therapy, the toxic side effects of an antitumor compound unavoidably interfere with its inhibitory activity on tumor cells, with the result that the drug-resistant cells appear. The delicate balance existing between the antitumor action of a drug, as powerful as it might be, and its injurious influence upon the animal and man organism, make the present day chemotherapy of cancer of doubtful value. Mitomycin C offers a striking illustration in this respect. Its antitumor activity is much greater, and its scale of activity much larger than those of any other known drugs. Yet the clinical application of Mitomycin C met with questionable success. It causes many serious side effects. It was not sufficiently effective in any case of an

CHART I



THE NUMBER OF ASCITES TUMOR CELLS
Count taken on 8th days after initial cell inoculum of about 2,000,000 cells
The treatment with drugs started four to five hours after the inoculum.
Consecutive therapy.

Ehrlich carcinoma ascites

I. Control, no treatment
II. Mitomycin C, 2.5 mg./kilo wt./ day for 7 days
III. Mitomycin C, 2.5 mg./kilo wt./ day for 6 days
IV. 6-AN, 2 mg./kilo wt. for 8 days
V. ETEE, 30 mg./kilo wt. for 6 days
VI. 8-azaguanine, 150 mg./kilo wt. for 6 days
VII. Methotrexate, 1.8 mg./kilo wt. for 6 days
VIII. 6-Mercaptopurine, 40 mg./kilo wt. for 6 days
IX. 6-Thioguanine, 2.5 mg./kilo wt. for 6 days
X. Methioprim, 300 mg./kilo wt. for 6 days

Sarcoma 180 ascites

1. Control, no treatment
2. Mitomycin C, 2.5 mg./kilo wt./ day for 7 days
3. Mitomycin C, 2.5 mg./kilo wt./ day for 6 days
4. 6-AN, 2 mg./kilo wt. for 8 days
5. ETEE, 30 mg./kilo wt. for 6 days
6. 8-azaguanine, 150 mg./kilo wt. for 6 days
7. Methotrexate, 1.8 mg./kilo wt. for 6 days
8. 6-Mercaptopurine, 40 mg./kilo wt. for 6 days
9. 6-Thioguanine, 2.5 mg./kilo wt. for 6 days
10. Methioprim, 300 mg./kilo wt. for 6 days

advanced tumor. This discrepancy between the animal experimentation and clinical observations can easily be understood. As we demonstrated in our present study, Mitomycin C was very effective when the therapy was started soon after the ascites cells were inoculated. It was much less effective when Mitomycin C was applied two or three days after the inoculum. In many investigations on Mitomycin C and other drugs, the compounds were screened on the tumors in an incipient stage, whether it was an ascites tumor or a solid tumor or leukemia. Thus the favorable reports published on the basis of these screening tests did not represent the true picture of the practical potentiality of such a drug, as far as its clinical application was concerned. Some investigators believe that the present day standard screening technique should be modified and that an antitumor drug should be submitted to a much more severe test, and screened on a more advanced animal growth.

For the last ten years, all the efforts of investigators have been concentrated on finding and on synthesizing various drugs. Yet very little attention, if at all, was given to the cytotoxicity of the drugs so discovered, and to possible measures which might decrease or minimize the side effects of chemotherapy. One of such measures, as reported in our present study, is the intermittent therapy, which might be used instead of the generally accepted consecutive administration of an antitumor drug. Although the antitumor drugs vary in their side effects, certain toxic manifestations are common to all of them. Leukopenia, hemorrhagic diathesis, and loss in weight are caused by almost all known antitumor drugs. Mitomycin C, nitrogen mustard and actinomycin induce, besides, shrinkage of the spleen in animals. Naturally, one may ask if there are any "protective measures" which might, if applied, decrease the gravity of the side effects caused by antitumor drugs, and which may render the chemotherapy of human cancer more effective and rational, than it is at present. It is this problem which is the subject of our current investigations, the results of which will be reported in our next publication.

Summary

The cytotoxicity of Mitomycin C was investigated on baby chicks, mice, and rats, and it was found that leukopenia, moderate neutropenia and lymphocytosis and loss of weight resulted from the consecutive

application of this antibiotic. The intermittent application of Mitomycin C resulted in lesser side effects.

Mitomycin C did not inhibit anaerobic glycolysis in tumor tissue.

Mitomycin C had no effect on the total PNA in cancerous tissue but decreased its total and nuclear DNA.

The Mitomycin C content of liver and spleen was estimated and an accumulation of it in these organs was observed.

Mitomycin C exerted a moderate inhibitory influence on mouse influenza virus PR8, and Rous sarcoma virus. It had no effect on baby chicks inoculated with Beard's lymphomatosis virus, Strain "R."

The effect of various doses and time of application of Mitomycin C on two ascites tumors, Ehrlich carcinoma and Sarcoma 180 were investigated. The intermittent therapy of Mitomycin C gave superior results as far as the side effects were concerned, than the consecutive treatment. With a total dose, 17.5 mg./kilo wt./mouse, administered intermittently, the survival rate was one hundred per cent.

The necessity of elaborating "protective measures" against the side effects of antitumor drug therapy was stressed.

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EFFECT OF AN ANTIVIRAL FACTOR ON ROUS SARCOMA AND CHICK LYMPHOMATOSIS VIRUSES*

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SOKOLOFF *et al.* (17) and Sokoloff and Kato (18) reported isolation of a substance, the product of enzymic activity, under certain conditions of fermentation, of *Penicillium brevi-compactum*, which was active against some viruses. They described this factor, named molasin, as a yellow substance, protein-free, of low toxicity, of a tentatively established formula $C_{27}H_{22}O_{12}S$, and melting point 72°C. Water soluble, it was easily oxidized, losing its biological activity when kept at room temperature. Its yield from the fermentation residue was very small, in fact too small for any large scale production or for clinical trials. The substance was, however, produced in a sufficient quantity for the laboratory investigation, and was tested extensively for its toxicity and biological activity. Mice, rats, and baby chicks manifested no toxic effect or loss of weight after three weeks of administration of a daily dose, 200 mg./kilo wt., injected intraperitoneally or subcutaneously. The factor did not affect adversely the blood forming system, and no destructive influence upon the bone marrow was observed. It induced, however, a mild lymphocytosis in rats and mice. It had no effect on malignant growth, either of an inhibitory or an accelerating nature. Molasin was found inactive against both gram-positive and gram-negative microorganisms. It showed a moderate inhibitory influence upon certain enzymes like hyaluronidase. Tested on polio virus in tissue culture HeLa cells, it gave a therapeutic index of 10. It was active against mouse influenza virus PR8 in chick embryo, and reduced the mortality rate of mice

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infected intranasally with the mouse adapted strain of virus by 60 per cent, with a daily dose of 2 mg./mouse/day, injected subcutaneously.

In the present study, the effect of this factor was investigated on Rous sarcoma virus and chick intravascular lymphomatosis.

Material and Technique

Rous sarcoma virus, originally obtained from the American Type Culture Collection, and chick lymphomatosis viruses, the strain RPL-12, preparation L-31, and the Beard's strain "R," both obtained from Dr. Ben Burmester, of the Regional Poultry Research Laboratory, U. S. Department of Agriculture, were used in the present study.

Two procedures were employed for evaluating the biological activity of molasin: (1) the method of intravenous inoculation into the chick embryo, and (2) the inoculation onto the chorioallantoic membrane.

The first assay, based on the intravenous method described by Polk *et al.* (14) consisted of intravenous injections of Rous virus or lymphomatosis virus to a chick embryo, 12 or 14-days old. Simultaneously, or shortly thereafter, or fifteen minutes before, molasin was inoculated, also intravenously to the same embryos. The embryo was killed at the intervals of 45 minutes, 2, 4, 6, 8, 12, 24, 48, and 72 hours and the vascular system, and various organs, particularly the spleen and liver, examined histologically. The embryos were fixed in 10% formalin, stained with hematoxylin-eosin, Giemsa and other stains. Some embryos were fixed in buffered osmium tetroxide for electron microscopy and photography. Other embryos were fixed in 4% lead subacetate or methyl alcohol and stained with 0.5% aqueous or alcohol solution of toluidine, for mast cells and metachromasia. Feulgen reaction was used for DNA, following the procedure described by us previously (19). The method of Alfert and Geschwind (1) for nuclear basic protein, and Heidenhain's hematoxylin for inclusion bodies and mitochondria were used. One half of the embryos were left to be hatched and the survival time and mortality rate of baby chicks, from the control and treated embryos, were recorded.

The second assay was described by us in our previous publication (Sokoloff *et al.*, 19) and is based on the technique recommended by Burnet and Ferry (8).

In the case of chick lymphomatosis virus, Beard's strain "R" was used for intravenous inoculations into chick embryo. The lymphomatosis virus, strain RPL-12, preparation L-31 was tested *in vivo* on the baby chicks susceptible to the virus and supplied by Dr. Burmester. The virus was inoculated intravenously to 11-day-old chicks, 0.2 ml. The treatment with molasin was started 24 hours after the virus inoculation, 100 mg./kilo wt./day/chick, injected subcutaneously. The survival time and life span of both groups, control and treated, were recorded.

The biological assay on Rous virus, with a "minimal infective dose" as recommended by Carr and Harris (9), Ponten (15) and others was occasionally employed by us. But in this study, we depended mostly on the intravenous inoculation method of assaying the antiviral activity of molasin.

Experimental

Rous Sarcoma Virus

Two aspects of Rous virus proliferation in embryonic tissue, when it is inoculated intravenously, deserves attention: the hemorrhagic diathesis and inclusion bodies. Duran-Reynals (11) described the hemorrhagic disease induced by Rous virus in baby chicks. This was confirmed by many other investigators working with Rous virus. But only recently, Bonson and Bang (4) reported that a similar hemorrhagic phenomenon was present in chick embryo inoculated intravenously with Rous virus. They gave much attention to the extravascular hemorrhage but did not describe the hemorrhagic manifestations in the blood vessels of the infected chicks.

Several investigators reported virus-like particles in Rous sarcoma cells. Claude *et al.* (10) observed round, dense particles, occurring in pairs or clusters, in the cytoplasmic portions of cells cultured from two different tumors. Oberling *et al.* (13) and Bernhard and Oberling (2) were unable to confirm these findings. Later, Bernhard *et al.* (3) reported the presence of virus-like particles in Rous sarcoma cells cultivated in vitro. Gaylord (12) described virus-like particles in the sections of two Rous sarcoma tissues. The particles, numerous in younger tumors, occurring singly or in pairs, were found on the outer surface of the cell membrane. The particles were round and consisted of thin membranes and a dense central mass. Some of the particles appeared to be vacuolized. No more recent reports on the

inclusion bodies or virus-particles of Rous virus have been published so far.

Intravenous inoculation method: A. Control. When Rous virus, dilutions 10^{-1} or 10^{-2}, 0.1 ml., were inoculated intravenously into the twelve-day-old chick embryos, the viral invasion was detectable 45 minutes thereafter. The chief pathologic feature was the appearance of very small hemorrhagic areas, intercellularly located, in the endothelium of the blood vessel wall. The size of hemorrhage depended on the dilution of the virus. At the dilution 10^{-1}, many embryos showed their presence, and the hemorrhagic spots extended to the tunica intima. At the dilution 10^{-2}, a few erythrocytes were found in some embryos, while the others were free of them. At a lower dilution, the tunica intima showed no sign of any pathologic alteration.

Forty-five minutes after the virus inoculation, some endothelial cells appeared to be swollen, with mitochondria slightly increased in size. Small, elongated bodies, possibly virus-like particles were visible in some swollen cells in the vicinity of the nucleus. In a few cells, they were quite numerous, while in others only two or three particles were detected. Their diameter was about 50 mμ.

Two hours after inoculation, these bodies were no longer found. The number of swollen cells in the endothelium was increased, and the hemorrhagic intercellular areas were observed in the tunicas intima and media at all dilutions of virus.

Six hours after inoculation, many endothelial cells appeared grossly swollen and vacuolized. The small hemorrhagic areas were detected in the tunicas media and adventitia, with some cells partially destroyed or vacuolized.

By twenty-four hours, all the tunicas showed pathologic alterations, multiple small intercellular hemorrhagic areas, eosinophilic infiltration and mitochondrial activity.

Forty-eight and seventy-two hours after inoculation, extravascular small lesions composed of neoplastic cells were visible, with blood vessels swollen, hemorrhagic extravascular areas nearby the vessels and capillaries (Photos 1-5).

After twenty-four hours, both the liver and spleen appeared to be normal, at the virus dilution 10^{-2}. With a higher dilution, 10^{-1}, a slight proliferation of hepatic cells was noticed. Forty-eight hours after virus inoculation, the spleen showed no significant histologic altera-

PHOTO 1.

Rous virus 10^{-1}, 0.1 ml. inoculated intravenously to 12-day-old chick embryo. 45 minutes after virus inoculation. Blood vessel wall. In some cells of the wall, virus-like particles are visible. × 6000

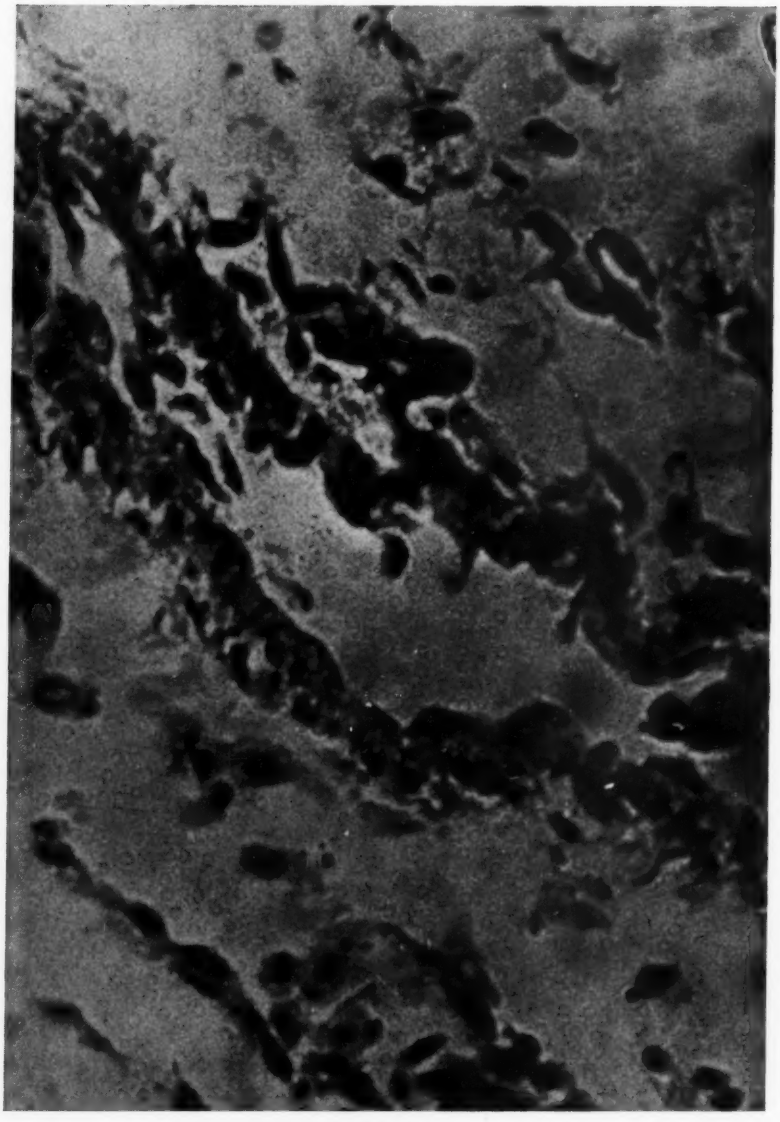

PHOTO 2.

Rous virus, 10^{-1}, 0.1 ml. inoculated intravenously to 12-day-old chick embryo. 6 hours after inoculation. The endothelial cells swollen, and some are vacuolized. No signs of virus-like particles. × 400

PHOTO 3.

Rous virus, 10^{-1}, 0.1 ml. inoculated intravenously to a 12-day-old chick embryo. 12 hours after inoculation. Infiltration of leukocytes, proliferation of parenchymal cells, intracellular hemorrhagic areas in the tunicas. × 200

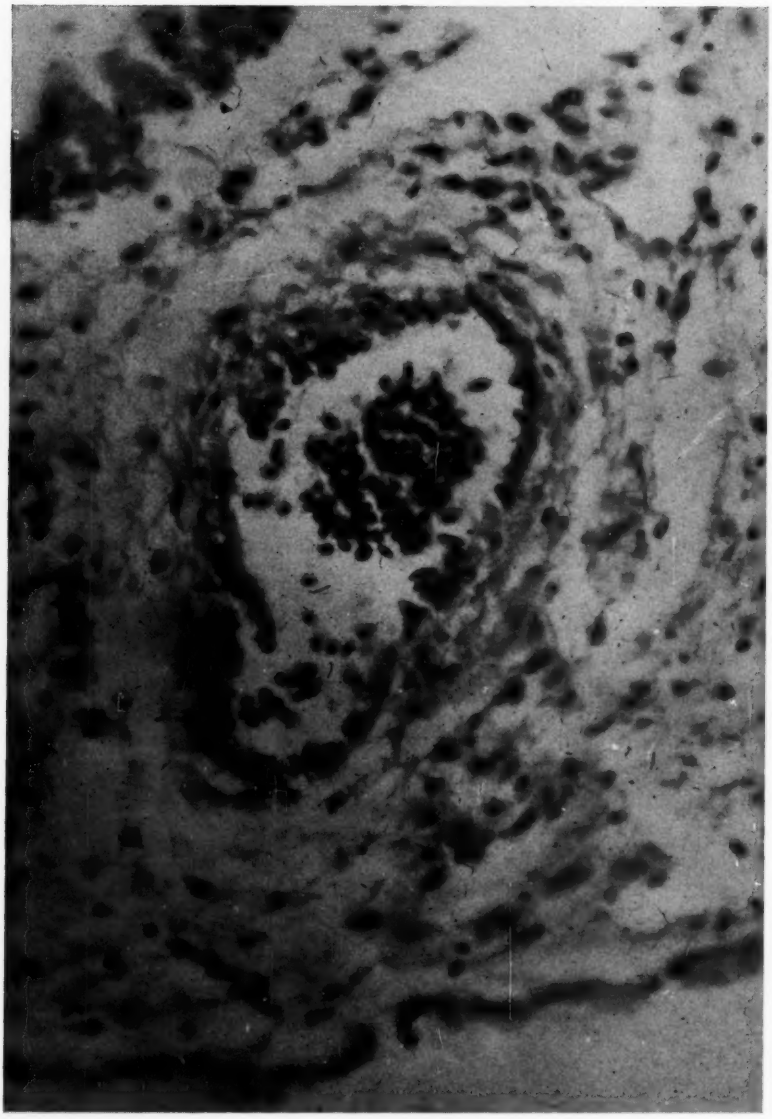

PHOTO 4.

Rous virus, 10^{-2}, 0.1 ml. inoculated intravenously to a 12-day-old chick embryo. 24 hours after inoculation. Swelling of tunical cells with partial vacuolization. Extravascular erythrocytes. × 400

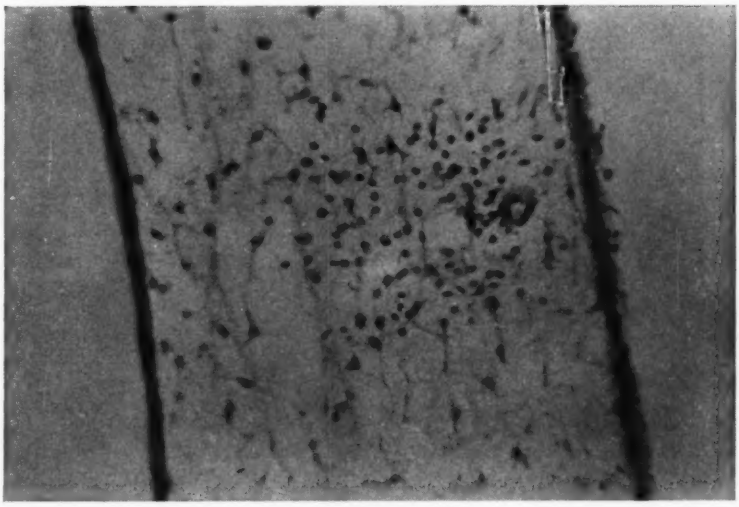

PHOTO 5.

Rous virus, 10^{-2}, 0.1 ml. inoculated intravenously to a 12-day-old chick embryo. 72 hours after inoculation. Membrane. Capillary. Erythrocytes and a few spindle-shaped tumor cells. × 430

tions, but the liver underwent considerable changes. Some hepatic cells and Kupffer cells were swollen, mitochondria increased in size, and the hepatic parenchyma showed a slight proliferation. At the dilution 10^{-1}, a few spindle-shaped cells, with granulated cytoplasm were observed on the edge of the hepatic parenchyma. Small groups of erythrocytes were visible in the parenchyma, located intercellularly. After seventy-two hours, many hepatic cells were considerably swollen, and vacuolized. The number of intercellular erythrocytes and spindle-shaped cells was increased and on the edge of hepatic tissue appeared large fibroblast-like cells, slightly vacuolized and with round-shaped nucleus showing caryolysis. The hepatic lobuli contained proliferated macrophages and erythrocytes. The splenic *lymphonoduli lienis* was not distinct and was considerably swollen (Photos 6-8).

The percentage of chicks, hatched at the same temperature (Jamesway incubator, 100.5° ± 0.5°F.) varied depending on the dilution of virus inoculated to the embryos. At the dilution 10^{-1}, about 45-48 per cent of the embryos were dead before hatching. At the dilution

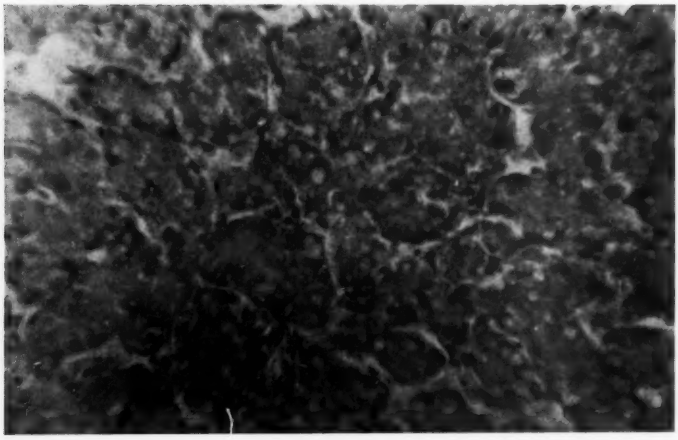

PHOTO 6.

Rous virus, 10^{-2}, 0.1 ml. inoculated intravenously to a 12-day-old chick embryo. 48 hours after inoculation. Liver. In the hepatic parenchyma some cells are vacuolized with extracellular erythrocytes present. Particularly at the edge of the hepatic tissue. × 430

PHOTO 7.

Rous virus, 10^{-2}, 0.1 ml. inoculated intravenously to 12-day-old chick embryo. Liver. 72 hours after inoculation. Spindle-shaped tumor cells on the edge of the hepatic parenchyma. × 430

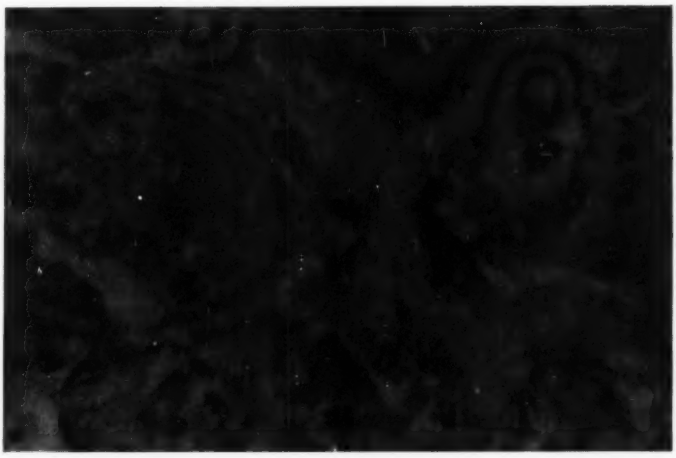

PHOTO 8.

Rous virus, 10^{-2}, 0.1 ml. inoculated intravenously to 12-day-old chick embryo. Liver. 72 hours after inoculation. Abnormal cells, with round shaped nuclei, showing caryolysis. Cell vacuolation. × 470

10^{-2}, the percentage of hatched chicks was an average 75 per cent. The mortality rate among baby chicks, hatched from eggs infected with Rous virus, varied from 92 to 75 per cent depending on the initial dilution of virus. Table I presents a summary of the average span of life and mortality rate of baby chicks hatched from the embryos inoculated with Rous virus, 10^{-2}.

TABLE I

MORTALITY RATE AND SPAN OF LIFE OF HATCHED CHICKS

Rous virus 10^{-2}, 0.1 ml.

Percentage of hatched chicks: 75

Number of hatched chicks: 59

	Survival Time, Days						
	3	5	7	9	11	13	Total
No. of Chicks Died	2	8	10	13	12	—	45
No. of Chicks Survived	—	—	—	—	—	—	14*
Percentage Survival							23.9

* Survived and remained alive for 63 days.

The dead chicks showed hemorrhagic areas and lesions in the liver and other organs.

B. Administration of Antiviral Factor. The inhibitory activity of molasin against Rous virus proliferation depended on the dose and the time of its application. A dose, 2 mg./0.1 ml. inoculated simultaneously or immediately after virus inoculation considerably inhibited virus invasion. Six and twelve hours after inoculation, the changes in the blood vessel wall were insignificant and hardly detectable. A few erythrocytes, located intercellularly in the endothelium, were all that was observed. The tunicas appeared to be normal. At twelve and twenty-four hours, small alterations were detected in the endothelial cells, some of which were slightly swollen, and occasionally intercellular erythrocytes in the tunicas intima and media. At seventy-two hours, some endothelial cells were vacuolized, and occasionally partially destroyed. Neither extravascular hemorrhages nor sarcomatous lesions were evidenced. At forty-eight hours, the liver and spleen showed no pathology. At seventy-two hours, proliferated hepatic cells, slightly vacuolized were observed in the liver, mostly on the edge of hepatic tissue. Erythrocytes were present in the hepatic parenchyma and interlobular connective tissue. In the splenic parenchyma, proliferated erythrocytes were present in trabeculas and at the edge of splenic tissue. Lymphonoduli were clear.

At the dose 3 mg./0.1 ml., the pathologic alterations in the blood vessel wall, liver and spleen were minimal, and consisted of occasional intercellular erythrocytes, visible in the endothelium. The hepatic and splenic parenchyma at 72 hours was slightly altered, with some cells swollen and vacuolized.

The survival rate of the chicks hatched from the embryos infected with Rous virus, 10^{-2}, and treated with 3 mg./0.1 ml. molasin, was 68 per cent against 25 per cent for the control group.

When the antiviral factor, 3 mg./0.1 ml. was inoculated fifteen or twenty minutes *before* the virus inoculation, its inhibitory activity was much less pronounced. During the first six hours, the retardation of viral invasion was quite noticeable, but after seventy-two hours, the extravascular hemorrhages and the lesions were much in evidence. The liver showed the spindle-shaped cells and considerable proliferation of hepatic parenchymal cells. The survival rate for the chicks hatched from the infected embryos was significantly smaller. Table III gives the summary of one of the tests.

TABLE II

MORTALITY RATE AND SPAN OF LIFE OF HATCHED CHICKS

Rous virus, 10^{-2}, 0.1 ml., inoculated immediately before 3 mg./0.1 ml. molasin was administered intravenously to 12-day-old chick embryo.

Number of hatched treated chicks: 48
Number of hatched control chicks: 40

	Survival Time, Days							
	3	5	7	9	11	13	15	Total
No. of Treated Chicks Died	—	—	—	—	5	7	3	15
No. of Treated Chicks Survived	—	—	—	—	—	—	—	33*
No. of Control Chicks Died	2	4	6	7	7	4	—	30
No. of Control Chicks Survived	—	—	—	—	—	—	—	10*
Percentage Survival of Treated								68
Percentage Survival of Control								25

* Survived and were alive for 137 days.

TABLE III

MORTALITY RATE AND SPAN OF LIFE OF HATCHED CHICKS

Rous virus, 10^{-2}, 0.1 ml., inoculated fifteen-twenty minutes after 3 mg./0.1 ml. molasin was administered intravenously to 12-day-old chick embryo.
Number of hatched chicks: 48

	Survival Time, Days						
	3	5	7	9	11	13	Total
No. of Chicks Died	—	—	—	12	12	—	24
No. of Chicks Survived	—	—	—	—	—	—	24*
Percentage Survival							50

* Survived and were alive for 123 days without sign of viral infection.

Comment: The results of assaying molasin by the intravenous method of Rous virus inoculations to chick embryos may be briefly summarized as follows:

(a) The small bodies, possibly the virus-like particles, were visible only on the slides of chick embryo taken 45 minutes after Rous virus inoculation. They were observed in a relatively few endothelial cells, were located intracellularly in the vicinity of the nucleus. Two hours after virus inoculation, no virus-like particles were detected. Possibly they were combined with protein or lipid molecules.

(b) Hemorrhagic disease was apparent in the chick embryos inoculated intravenously with Rous virus. Erythrocytes in various numbers were visible, situated intercellularly, in the endothelium and the tunicas of the vascular wall. After forty-eight and seventy-two hours, hemorrhagic areas extended to the extravascular region of the embryo and the sarcomatous lesions appeared in the liver and spleen.

(c) The chicks hatched from the embryos inoculated intravenously with Rous virus showed hemorrhage and sarcomatous lesions in the liver and other organs.

(d) The antiviral factor, molasin, inoculated intravenously, simultaneously or immediately after virus inoculation, inhibited considerably but not completely viral proliferation. When the factor was inoculated fifteen or twenty minutes before viral inoculation, its inhibitory activity was much less pronounced.

Chorioallantoic membrane method. The Rous sarcoma virus, from the American Type Culture Collection, diluted in Simms Salt solution, dilutions 10^{-1} and 10^{-2}, 0.1 ml. was inoculated onto the chorioallantoic membrane of a ten-day-old chick embryo. Molasin, 1.0, 2.0 and 3.0 mg./0.1 ml./egg was also injected onto the membrane at various times: fifteen minutes before, fifteen and thirty minutes after virus inoculation. On the seventh day after the inoculation, the eggs were opened and the number of lesions and their size were recorded. Each test covered 50 eggs, 25 control and 25 treated. The results of these tests are presented in Table IV.

The data of Table IV shows that the factor, molasin, inhibited Rous sarcoma virus when the chorioallantoic membrane technique was used. The inhibition was not complete, and was most effective at the virus dilution 10^{-2}, and 3 mg. of the factor, applied fifteen minutes before or after the virus inoculation, reducing the number of lesions by 73.5%. The inhibitory effect was approximately the same when the factor was administered before or after the virus inoculation. In both instances, the number of lesions was reduced considerably compared with that of the control. The size of the lesions was smaller in the treated than in the control embryos. Medium and large tumors were observed in the control embryos at the virus dilution 10^{-1}. They were absent in the treated groups. All in all, the results of these tests with the chorioallantoic membrane technique, correspond to those obtained with the intravenous inoculation method, indicating the inhibitory influence of molasin on Rous virus.

TABLE IV

EFFECT OF THE ANTIVIRAL FACTOR ON ROUS SARCOMA VIRUS

Chorioallantoic membrane technique. Rous virus, 10^{-1} and 10^{-2}, 0.1 ml.

Molasin: 1, 2, and 3 mg./0.1 ml. saline, injected before or after virus inoculation.

Each test: 25 control and 25 treated eggs.
Embryos: 10-day-old

Inhibition is estimated as percentage of the activity of the control virus, which was taken to be one hundred per cent. In the control series, 0.1 ml. saline, containing 200 units of penicillin, was injected instead of molasin.

Dilution, Rous virus 0.1 ml.	Inhibition Percentage Concentrations of the antiviral factor 0.1 ml.		
	1.0 mg.	2.0 mg.	3.0 mg.
10^{-1}			
15 minutes before	34.5	38.3	41.3
15 minutes after	32.4	41.0	42.5
30 minutes after	22.2	26.5	28.4
10^{-2}			
15 minutes before	56.7	65.6	73.5
15 minutes after	59.2	63.4	72.5
30 minutes after	43.2	49.7	56.6

Chick Lymphomatosis Virus

In 1946, Burmester *et al.* (5) reported the isolation of a filtrable agent producing lymphoid tumors in chickens. In more recent publications, Burmester *et al.* (6, 7) described the pathogenesis of the viral Strain RPL-12, which induces intravascular lymphomatosis in chicks in about 30 to 130 days after inoculation. They recognized this pathologic entity as erythroblastosis. The typical visceral lymphomatosis occurs about 120 days after inoculation. During the period October 1957-April 1959, six batches of baby chicks susceptible to this virus were shipped to us by Dr. Burmester, and inoculated with the Strain RPL-12, preparation L-31, 0.2 ml. Log 4. One-half of these chicks, totaling 343, were treated with the antiviral factor, while the rest served as control. The virus was inoculated intravenously to 11 or 12 day old chicks. The factor, molasin, was administered subcutaneously, 100 mg./kilo wt./day/chick for various periods of time, but with the same total dose, 1.5 gm./kilo wt./chick, in all experiments. The mortality rate and survival time of the control and treated groups are given in Table V.

In our first tests with the antiviral factor applied to chick lympho-

TABLE V

EFFECT OF THE ANTIVIRAL FACTOR ON CHICK INTRAVASCULAR LYMPHOMATOSIS
Virus: RPL-12, preparation L-31, 0.2 ml., Log 4.
Chicks, 11-12 days old, inoculated intravenously, total number: 343
Molasin: 100 mg./kilo wt./day/chick. Total dose: 1.5 gm./kilo wt./chick*

Batches Number	Number Chicks	Period Observation Days	Mortality Rate Percentage	Average Survival time, days, for infected chicks
I. 12-19-57 Control	19	110	91	51
II. 12-19-57 Treated	19	110	75	61
III. 12-21-57 Control	22	109	95	55
IV. 12-21-57 Treated	20	109	60	64
V. 3-24-58 Control	29	150	85.5	46
VI. 3-24-58 Treated	29	150	55.5	71
VII. 3-29-58 Control	25	121	80	54
VIII. 3-29-58 Treated	25	121	48	73
IX. 4-14-58 Control	40	133	67.4	55
X. 4-14-58 Treated	40	133	41.5	60
XI. 10-23-58 Control	21	119	71.5	66
XII. 10-23-58 Treated	27	119	25.9	77
XIII. 10-23-58 Treated** Antiviral Factor and Mitomycin C	27	119	22.2	71

* Batches II and IV, the factor administered contained 25% impurity.
Batches VI, VIII, X, XII, and XIII, the factor used was in a more pure form, containing 11% impurity.

** The Batch XIII received, besides the factor, total dose 1.5 gm./kilo wt./chick, also Mitomycin C, total dose 16 mg./kilo wt./chick.

matosis, which were started in October 1957, it was in a form containing about 25 per cent of impurity. It was less active than the compound used in the more recent assays. Accordingly, the results of treatment with molasin on the infected chicks, in batches II and III, were not very encouraging, as one may see from Table V. Gradually, we were able to obtain molasin in a more pure form and more biologically active. Beginning with batch VI, the purified form of molasin, containing 11% impurity was used. Nevertheless, for the sake of records, we included in the final total tabulation, the data

from all the batches treated with molasin. It appears that this factor had considerable inhibitory influence on the course of chick intravascular lymphomatosis, reducing the mortality from 86.5 control, to 46.8 per cent, treated (the total figures). If we analyze the results of individual series, like those of batches XI and XII, when the purified molasin was administered, decrease in the mortality rate was even greater: 71.5 for control; 25.9 for treated. In all experiments presented on Table V, molasin was applied intermittently, three times a week. Our tests, which are not included in Table V, with the consecutive administration of molasin, total dose 1.5 gm./kilo wt./chick, in the first fifteen days after virus inoculation, have given results inferior to those obtained with the intermittent therapy.

In one series of experiments, batch XIII, molasin, was combined with Mitomycin C, total dose 16 mg./kilo wt./chick. The additive effect of Mitomycin C was negligible. The mortality rate was reduced only from 25.9 (molasin alone) to 22.2 per cent (molasin and Mitomycin C). There was considerable loss in weight among the chicks of this batch (38 per cent), with eczematous infection of the skin and occasional alopecia seborrheica.

While the mortality rate of chicks infected with the virus, Strain RPL-12, was brought down considerably by administration of molasin, there was only a slight difference in the survival time of the control and treated chicks, which died from the infection.

The Strain "R," Beard's virus is more virulent than the strain RPL-12. It kills chicks in about three weeks after inoculation. We investigated the Strain "R," which was also supplied to us by Dr. Burmester, by the method of intravenous inoculation to chick embryos. The virus, diluted x 5, 0.1 ml. in Simms salt solution, was inoculated intravenously to fourteen-day-old embryos, which were killed at the intervals of 45 minutes, 2, 4, 8, 12, 24, 48, 72, and 96 hours. There was a striking difference in the invasion pathways between Rous virus and lymphomatosis virus. While considerable destruction to the vascular wall was caused by Rous virus, no sign of any damage to the endothelium or to the tunicas of the wall was detected at any time of invasion. The slides of the vascular wall made after 24, 48, and 72 hours, showed no pathologic alterations in it, except a slight eosinophilic extravascular proliferation. Apparently the virus passed through the circulatory system, without affecting it, to the organs of the embryo.

In the liver, at the virus dilution x 5, the first signs of viral invasion were apparent at twelve hours. In two embryos, a few hepatic cells showed swollen nuclei containing small inclusion bodies, with an increased cytoplasmic density and enlarged mitochondria. The inclusion bodies were of various sizes and forms, occasionally forming basophilic blocks. The nuclear sap gave positive Feulgen and tetrozolium reactions.

At twenty-four hours, the number of inclusion bodies was considerably increased and they were visible in most of the embryos. In a few embryos, round large cells with a clear nuclei were detected. These abnormal cells were found mostly in the extravascular space.

At forty-eight hours, the inclusion bodies were no longer visible and the number of proliferated, round, large cells was increased. In some livers, the sign of sclerotic process was noted.

At seventy-two hours, the proliferative lesions were increased in size, and some of these cells were transformed into fibroblast-like cells. The hepatic parenchyma was slightly edematous, with eosinophilic infiltration present.

The pathologic alterations in the spleen became evident only after forty-eight hours. Large cells with clear nuclei were observed in many embryos. We were unable to detect inclusion bodies in the splenic cells at any time after virus inoculation.

The virus, Strain "R," dilution x 5, was very injurious to the chick embryos. Although many of them remained alive until the day of hatching, the large proportion of hatched chicks were dead, or lived one or two days thereafter, showing gross lesions in the liver.

When molasin, 3 mg./0.1 ml./embryo was inoculated intravenously, simultaneously or immediately after virus inoculation, the liver of the embryos did not show any nuclear inclusion bodies at twenty-four or forty-eight hours, but they were detected at the seventy-two hour examination in some embryos. The hatched chicks appeared to be more lively. Forty-eight per cent of hatched chicks died during the first six days, with an average survival time of 4 days. Fifty-two per cent of hatched chicks survived and did not show any sign of infection for the eight following weeks. Apparently, molasin inhibited to a considerable degree, but not completely, the virus, Strain "R," inoculated to chick embryo.

Discussion

The property of Rous virus to induce hemorrhagic disease, which was emphasized by the work of Duran-Reynals (11), Bonson and Bang (4), Shrigley (16) and others, was evidenced in our present study with the intravenous inoculation method on chick embryos. There was a striking difference in the cytopathology of the embryo vascular wall induced by Rous virus and lymphomatosis virus. In the first instance, hemorrhagic areas were present both in the endothelium and the tunicas of the wall shortly after virus inoculation. In the case of lymphomatosis virus, the hemorrhagic diathesis was absent. No hemorrhagic areas were observed in the embryo vascular wall at any time after virus inoculation. The interesting feature of molasin inhibitory influence on Rous virus was the almost complete absence of hemorrhage in the treated embryos. The embryos inoculated intravenously with Rous virus and treated with molasin showed no sign of any hemorrhagic phenomenon. The mechanism of the hemorrhage-producing property of Rous virus is not clear, and its physiologic nature is not defined as yet. The possible relationship between this property of Rous virus and its infectivity is of considerable interest and deserves further investigation.

Summary

A method of intravenous inoculation of Rous virus to chick embryos was described. The endothelial cells of the embryo blood vessel wall, 45 minutes after virus inoculation, showed small bodies, possibly elementary bodies of Rous virus. Two hours after inoculation, these bodies were no longer visible, either in the endothelium or in the tunica cells.

The chief feature of Rous virus invasion of the embryo blood vessel was intercellular hemorrhages, which after 72 hours extended to the extravascular areas, at which time sarcomatous cells appeared in the liver and other organs of the embryos.

The chick lymphomatosis virus, Beard's Strain "R," inoculated intravenously to chick embryos, caused no damage to the embryo blood vessel wall. Nuclear inclusion bodies of the virus were visible in hepatic cells twelve and twenty-four hours after virus inoculation. Large proliferated cells appeared in embryo livers, forty-eight hours after inoculation.

An antiviral factor, molasin, inhibited considerably but not completely, Rous virus, when inoculated simultaneously with it, or immediately thereafter. When inoculated intravenously twenty minutes before virus inoculation, molasin was less active.

The susceptible breed of chicks inoculated with chick intravascular lymphomatosis virus, Strain RPL-12, was treated with molasin, intermittently for five weeks, with a total dose of 1.5 gm./kilo wt./chick. The mortality rate of infected chicks was reduced from 86.5 for control to 46.8 per cent for the treated group.

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DIFFERENTIAL GROWTH OF NERVE CELLS IN CEREBRAL CORTEX*

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INTRODUCTION

QUANTITATIVE growth analyses of nerve cells are rare. Peters and Flexner (1950) investigated the frontal cortex of the guinea pig. They distinguished two growth phases of the nerve cell body volume; a fast and a slow phase. A sharp decrease in the cell density occurred during the fast growth phase. No analysis was made by the differential growth equation, but the growth of nerve cells was plotted against time. Flexner and his associates (1948, 1950, 1953) investigated also the development of enzyme systems in the cerebral cortex of the fetal guinea pig. Their classical studies have resulted in the definition of a "critical development period." This period is on the one hand enzymatically characterized by an increase in activity of apyrase, succinic dehydrogenase, cytochrome oxidase and other enzyme systems, and on the other histologically by the development of processes and the cessation of the nuclear growth of nerve cells.

Noback and Moss (1956) analyzed the growth of human brain by differential growth equations. They determined the ratios of growth of 14 linear dimensions. In general they found the gradients of growth constantly proportional from the second fetal month to the adult stage. Recently reports have been published on differential growth equations of some human fetal cranial bones (Moss, 1955) and of the rat skull (Moss and Baer, 1956).

Numerous data from the older literature have been compiled by Ariëns Kappers (1943, 1944) on the relationships between body weight and brain weight. In many species a logarithmic relationship could be calculated.

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It was found previously (Schadé, 1959 a, b) that in the rabbit the relationship between body weight on the one hand and brain weight and pallial weight (cerebral cortex with underlying white matter) on the other during development can be expressed as a power function. Three growth phases of the postnatal pallial weight could be recognized, which tentatively were correlated with the histological development of the cerebral cortex.

A sharp decrease in the density of nerve cells of the cerebral cortex of the rabbit was observed after birth (Schadé, 1959c). During the first 10 postnatal days a drop in the cell density from 55 to 9.9 x 10^4 per cubic mm was measured; after this period the rate decreased up to 30-35 days after birth. At that time the adult value of cell density was reached.

This paper deals with a quantitative analysis of the postnatal development of the nerve cell body volume and the nuclear volume in the cerebral cortex of the rabbit. Attention was paid also to the development of the Nissl bodies in the nerve cells of the cerebral cortex.

Methods

For the study of size of the nerve cells and the nucleus the histological sections were stained with toluidin blue or thionin. Swelling of the cell bodies, which occurs a few minutes after circulatory arrest, (Van Harreveld, 1957) was avoided as follows. One hemisphere was widely exposed and the dura removed under ether narcosis. The activity state of the cerebral cortex was checked with the electroencephalograph. During normal EEG activity cold isopentane (—160°C) cooled to its freezing point with liquid nitrogen was poured on the cortex. The head was then severed from the body and quickly immersed in cold isopentane. After a few minutes the head was placed in isopentane at a temperature of —30°C to —35°C. The isopentane was rinsed off with absolute alcohol of the same temperature. The head was then placed in a beaker with absolute alcohol at a temperature of —25°C for a week. Slices of the cortex were removed, dehydrated, embedded in paraffin and stained with toluidin blue or thionin. The freezing method was adopted to maintain the fluid distribution between cellular and extracellular compartments.

The dimensions of the perikarya and the nuclei were measured in cortical strips, 300 μ wide. All measurements were done in the same

cortical field as both size of the nerve cells and the density differ considerably in the various architectonic fields of the same species. The various cortical fields are difficult to recognize however in the cerebral cortex of the young rabbits. For this reason we did our measurements in the mid-sagittal plane of the cerebral cortex. Only the middle part of the sections was used for the determinations. Two dimensions were taken for each perikaryon and each nucleus, the maximum length (a) along the length direction of the nerve cells and the maximum width (b) perpendicular to this direction. The dimension perpendicular to the plane of the section was considered to be equal to the width of the perikaryon. Only sections of whole perikarya and whole nuclei were measured. The volume of the nerve cell body and the nuclei was calculated by treating the elements as ellipsoids using the formula $1/6\ \pi\ ab^2$.

Results

Three quantitative histological values of the developing cerebral cortex of the rabbit have been determined: the average volume of the nerve cell bodies, the average nuclear volume and the number of Nissl bodies.

The volume of the nerve cell bodies during development of the cerebral cortex. The measurements of four animals of each age (mean values with standard error of 200 nerve cell bodies for each animal) are represented in the form of a graph, in which the logarithms of the cell body volumes are plotted against the logarithms of the body weights (Figure 1). In 2-day old animals a mean volume of the cell bodies of 272 μ^3 was found. A fast increase of the cell body volume occurred up till about 10 days after birth. At this age a cell body volume of 715 μ^3 was found. After this age a decrease in rate of growth of the cell body could be observed. At 21 days after birth a mean volume of 920 μ^3 was measured for the cell body volume. At 30 days after birth a mean value of 1156 μ^3 was measured (Fig. 2) which did not differ statistically from the mean value of the cell body volume in the adult animals. Two distinct periods with a different rate of growth can be recognized. During the first period (0-10 days after birth) a mean increase of the cell body volume of 54 μ^3 can be calculated, however during the second period (12-30 days after birth) a mean increase of only 22 μ^3 can be observed.

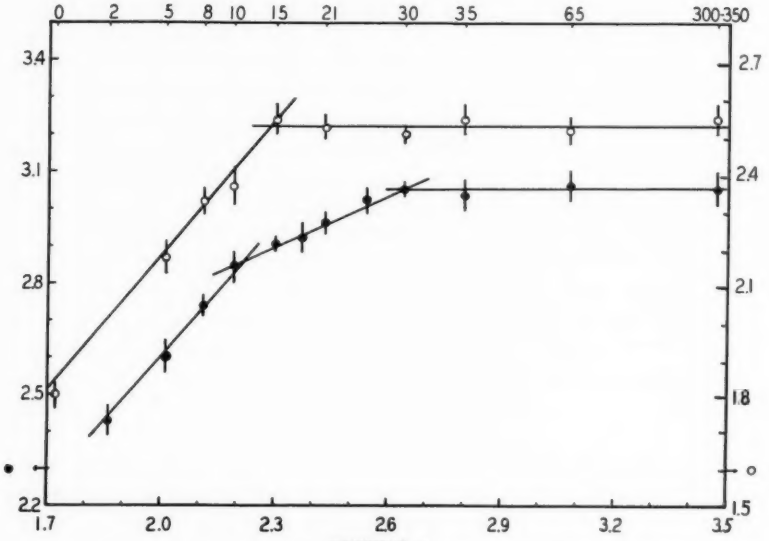

FIGURE 1

The development of the nerve cell body volume (·) and nuclear volume (°). On the left ordinate is plotted the log. of the mean volume of the nerve cell bodies in μ^3 and on the right ordinate the log. of the mean nuclear volume in μ^3. The log. of the body weight is plotted on the lower abscissa; the upper abscissa represents an alogarithmical time scale. The vertical line through each point indicates the standard error.

As was found previously (Schadé, 1959a) the relation between body weight on the one hand and brain weight and pallium weight on the other during growth (in the rabbit) can be expressed as a power function ($y = ax^b$). The theory of this differential growth equation is discussed by Huxley (1932), Zuckerman (1950) and others. In this function -y- is the dimension of an organ and -x- the body weight. The factor -a- has no biological significance. The factor -k- is the ratio of specific growth rates; it remains the same for certain phases in the development but changes at definite points. Such a change takes several days. It is known as an interphase or critical development period.

The postnatal development of the nerve cell body volume shows three periods of growth separated by two interphases. The first interphase occurs when the mean body weight is 175 gm., which is approxi-

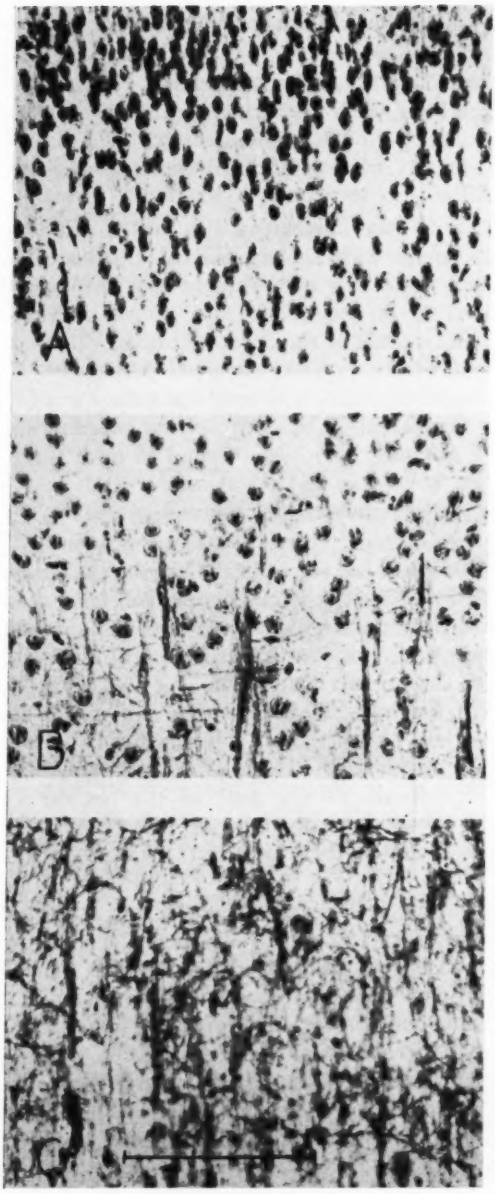

FIGURE 2

Micrographs of Gallocyanin-Cajal stained preparations.
A: 2 day old animal.
B: 10 day old animal, deep cortical layer.
C: 20 day old animal, layer II/III.
Notice in B and C the marked increase in size and number of the dendritic plexuses.
Calibration: 25 μ.

mately between 10 and 12 days after birth. The second interphase occurs when the body weight is 425 gm. (about 30 days after birth). The -k- of the first period is approximately 1.21, that of the second 0.45. The -k- factors were determined with the method of the least squares.

The results are in good agreement with those of Peters and Flexner (1950). They found with the method of Chalkley (1943) in the guinea pig a sharp increase in the nerve cell body volume from the 35th to the 43rd day of gestation. The rate of increase then diminished. The main increase of the nerve cell body volume in the guinea pig (Peters and Flexner, 1950) occurred prenatally. This may be explained by the long gestation time of the guinea pig (66 days); the gestation time for the rabbit is 30 to 32 days. Haddara (1956) using the method of Abercrombie (1946) found in mice three days old, a mean perikaryal volume of 281 μ^3, at an age of 7 days 535 μ^3, after which age no further increase was observed.

The size of the nuclear volume during development. The dimensions of the nuclei were measured in the same sections as used for the determination of the size of the cell body. The same dimensions were determined and the nucleus was also treated as an ellipsoid. Fig. 1 shows the mean value with its standard error. In 0-day old animals a mean value for the nuclear volume of 63 cubic micra was found. A fast increase of the nuclear volume occurred up till about 15 days after birth. At this age a nuclear volume of 349 μ^3 was calculated. This value does not differ statistically from the mean value in adult animals. The postnatal development of the nuclear volume shows two periods of growth separated by an interphase. The interphase occurs between 10 and 15 days after birth. The -k- of the first period is 1.19.

Apparently the nucleus reaches morphologic maturity during this interphase. Both the cell body and the nucleus go through a fast development phase; at the end of this period either the rate of growth decreases markedly (cell body) or stops completely (nucleus).

The Nissl substance during development of the nerve cells. Prior to the appearance of the Nissl bodies in the cytoplasma of the nerve cells a basophilia, which may persist during development, can be observed in many cells. Peters and Flexner (1950) defined "true" Nissl bodies as clumps of basophilic material. According to their

standards we distinguish in the nerve cells during development "dust-like basophilia" and "true Nissl bodies." Nissl bodies are first seen in relatively few cells in 10-day old animals (3 percent). A marked increase in the number of cells, having dustlike basophilia, occurs in 10-day old animals (Table I). The number of cells with Nissl bodies

TABLE I
CELLS SHOWING CYTOPLASMIC BASOPHILIA AND NISSL BODIES
(Examination of 250 cells for every age group)

Age in Days	Dustlike Basophilia	Per Cent	Nissl Bodies	Per Cent
0	15	6	0	0
10	115	46	7	3
15	102	41	81	32
20	85	34	130	52
30	80	32	158	63
Adult	86	34	152	61

increases very fast between 10 and 20 days after birth. The appearance of the Nissl bodies coincides with the fast increase in number and size of the cell processes (Schadé, 1959c) and the cessation of growth of the nucleus. Peters and Flexner (1950) found also a remarkable increase in the quantity of Nissl substance in a relatively short period (from the 41st to the 44th day of gestation in the guinea pig).

DISCUSSION

It was shown that the growth of the cell body volume and the nuclear volume could be analyzed by the differential growth equation. For both values a high ratio of specific growth rate was observed during the first 10-12 postnatal days. In the following 20 days of the development of the cerebral cortex the cell body volume increases with a much lower rate. However, the nuclear volume ceases to increase, and reaches between 10 and 15 days after birth a volume, which value is indistinguishable from the value found in adult animals. The nucleus reaches its morphologic maturity in this period.

Our observations suggest that a critical development period (Flexner, 1953) can be distinguished for the rabbit lasting from about 8 to 15 days after birth. In this period the interphases of both graphs occur.

A second critical development period can be observed from 30 to 35

days after birth. In this period the second interphase of the development of the cell body volume occurs.

The first critical development period is characterized by many striking morphological and functional events. The cytoplasm of the nerve cells undergoes many changes; a fast increase in the number of Nissl bodies can be observed and the dendritic plexuses augment in size and number, Fig. 2. The spontaneous electrical activity from the cerebral cortex develops also from 10 to 15 days after birth (Schadé, 1959a).

Electronmicroscopic investigations of nerve cells (Palade, 1953, 1955) reveal that the Nissl substance is the most impressive single architectural unit in the perikaryon. So called microsomes can be isolated from the Nissl bodies by differential centrifugation. These microsomes contain most of the cytoplasmic pentose nucleic acid and are rich in phospholipids. Probably ribonucleic acid is concentrated in the smaller particulates of the microsomes. Palay and Palade (1955) and Palay (1956) assume that the Nissl bodies in the nerve cells are the centers of protein production. The fast increase of the Nissl bodies in the perikarya and the dendrites would suggest a functional development during this period, characterized by the accumulation of protein producing elements. This is furthermore supported by the fact, that the concentration of RNA in developing embryonic tissue (Flexner, 1953) is proportional to the amount of protein synthetic activity carried out by the cells. A close parallelism probably exists between the functional maturation of the nerve cells (development of the spontaneous electrical activity) and the morphological maturation (appearance of Nissl bodies and fast outgrowth of dendrites).

Additional evidence is obtained from the observations of Jacobs and Snider (1949). They found typical cerebellar activity of 10-60 μV with a frequency of 150-250 per second in the rat for the first time at about the twelfth postnatal day. Nissl substance appeared about the tenth day after birth in the Purkinje Cells.

The period from 10 to 20 days after birth is in the rabbit also characterized by a histochemical development of PAS-positive material (Schadé, 1959c). The PAS-positive reaction is associated with a carbohydrate-protein complex which might resemble the nature of acetylcholine esterase. Although the function of the acetylcholine-system still is controversial, it is most likely that it is important for the normal function of the brain.

The above mentioned observations have led to the definition of a critical period in the development of the cerebral cortex of the rabbit, lasting from about 8 to 15 days after birth.

Summary

1. Increase in nerve cell body volume and nuclear volume during post-natal growth was investigated in the cerebral cortex of the rabbit. The growth of both parameters could be analyzed by differential growth equations.

2. The appearance of Nissl-bodies in the protoplasm of nerve cells and the growth of dendritic plexuses were also studied.

3. A critical period in the development of the cerebral cortex of the rabbit was defined.

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WATER FIXATION AS THE ULTIMATE CAUSE OF TISSUE GROWTH*

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SOMATIC growth is readily influenced by many agents: it can be inhibited by virtually all stressors, and conversely, numerous agents can stimulate the growth of the body as a whole or of its individual organs. STH (the somatotrophic hormone) is probably the most important general growth-stimulator of vertebrates, while other trophic hormones (e.g., ACTH, LH, FSH) are especially potent organ-specific growth-promoters. Yet, all these and the many other known growth-regulators (e.g., food factors, functional demands, "wound hormones," genetic predispositions) act only indirectly by modifying certain chemical reactions in the responsive tissues.

Nothing is known about the possible existence of any final common pathway in growth processes. The great selectivity with which certain cell-types respond only to specific growth-stimulators appears to speak against such an assumption. At first it is perhaps difficult to see how muscular work could act selectively on the exercised muscle, ACTH on the adrenal, or partial hepatectomy on the liver remnant, to stimulate the growth of such totally dissimilar tissues by virtue of any one common agency. Still, depending upon its pre-existent structure, each receptor cell could react to the same immediate growth-stimulus specifically, that is, by adding more of its own kind of living matter. STH can act as a growth hormone for all organs and, *in vitro*, the most varied tissues begin to proliferate in their new aqueous medium. These facts suggested to us that there may be some common, final growth-stimulator, a substance which could become effective diffusely in diverse structures (e.g., during embryonic life, under *in vitro* condi-

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tions or after STH-treatment) but which could also act with great selectivity on certain cell-types, thereby permitting their differential development.

Before undertaking a search for the hypothetical ultimate stimulus of tissue growth, it is well to survey the *postulates* which it would have to fulfill in order to be recognized as such. We feel that if such a substance exists it may be expected to exhibit the following characteristics:

1. *Topical activity.* Obviously, the immediate cause of growth should act where it is applied; it should not be effective only when introduced systematically, that is, only under conditions which permit action through the intermediary of some other organ.

2. *Particularly intense and rapid activity.* It may be expected that the immediate cause would stimulate growth of unusual intensity and without any lag-period, such as often occurs with agents that can exert their effect only through the intermediary of sluggishly reacting relay stations.

3. *Relatively unconditional activity.* The hypothetical agent should be less dependent upon "conditioning factors" than are indirect stimulators of growth whose effects depend on the maintenance of receptiveness in all their relay stations. Indeed, upon topical application, the immediate causative factor would presumably stimulate local growth, even during starvation, when systemic anabolism and cellular proliferation are inhibited elsewhere in the body. Certain local growth processes can take place in the absence of food-intake; the growth of neoplasms, the enlargement of the adrenals during the alarm reaction, or of the liver remnant after partial hepatectomy, are examples in point. Here, topical growth is presumably accomplished through some local factor which attracts building materials from other regions of the organism.

4. *Ubiquitous activity.* By definition, the substance in question would have to be capable of selectively accumulating and acting in any tissue, otherwise it could not be a common, ultimate cause of growth anywhere in the body. The glandulotrophic hormones, for instance, may stimulate their respective targets to attract or produce the hypothetical common growth-stimulator, but these substances themselves do not satisfy the postulates of such an immediate growth-promoter.

Our earlier studies had shown that the topical growth, that can be induced by the implantation of inanimate tissue-scaffoldings, is closely related to—if not dependent upon—fluid retention (2–4). It has long been known, furthermore, that very young and rapidly growing tissues are particularly rich in water, although here, hydration was regarded as a mere accompaniment or even a consequence rather than as the immediate cause of growth. In any event, it did not seem technically feasible to explore water as a possible topical growth-stimulus under *in vivo* conditions, because it is so rapidly absorbed that it could not exert any lasting effects at the site of administration. In the course of our studies with the "granuloma-pouch technique" (1), we found that water injected into a cavity completely enclosed by granulomatous tissue is held there due to the "phenomenon of inflammatory sequestration." However, here, conditions for the demonstration of a growth-stimulating effect are especially unfavorable, because the chemical or microbial inflammatory irritant itself stimulates intense fibroplasia in the connective tissue with which it comes in contact. This is precisely how the capsule is formed that sequesters water by preventing its further penetration into more distant connective-tissue layers and its eventual absorption into the blood. Hence, centrifugal growth of connective tissue beyond the capsule is prevented. Centripetal growth into the granuloma-pouch cavity is likewise impossible, because of the unfavorable metabolic conditions (great increase in osmotic pressure, enzyme content and acidity, etc.) that prevail within the exudate.

Here, we should like to report on experiments in which modifications of the granuloma-pouch- and tissue-scaffolding-techniques permitted us to keep an excess of water constantly in close contact with connective tissue. As we shall see, this resulted in an extraordinarily rapid and intense topical tissue growth which occurred even during starvation. On the basis of these and related observations, we shall attempt to show that of all the substances examined only water, the most plentiful and pharmacologically inert body-constituent, fulfills the postulates of an ultimate growth-stimulator.

Materials and Techniques

Experimental animals and their maintenance.—For all our experiments, female Sprague-Dawley rats, with an average initial body weight of 150 (range 140-160) g, were used. Each group consisted of

6 animals but, since all experiments were repeated at least twice and some three times, the groups listed in our table represent a minimum of 12 rats each. Throughout the period of observation, the animals were maintained exclusively on "Purina Fox Chow" (Ralston Purina Co. of Canada) and tap water.

Manufacture of tissue scaffoldings.—Only two types of tissue scaffoldings were used: the "ellipsoid spiral" and the "corkscrew spiral" type. The *ellipsoid spiral* is prepared by winding a No. 18 Chromel wire "A" (Fisher Scientific Co.) around a metal rod with a diameter of 15 mm. At this point the coils touch each other and completely cover the surface of the template with a single layer of wire. However, after removing the rod from this wire tube, the latter is stretched so that a looser spiral results with 15 rings per 5 cm. length. The spiral is now cut up into 5 cm. segments in which the last two or three rings on each end are further bent by rotating them inwards with a pair of fine pincers, so that a roughly ellipsoid structure results (Fig. 2). The rounded tapering ends obviate the danger of perforating the skin when the scaffolding is subcutaneously implanted, a complication which often occurs with ordinary spirals, if the cut ends come into direct contact with the derma.

Chromel "A" seems to be an inert alloy which produces no tissue reaction attributable to any chemical interaction with body fluids. Still, we have also performed control experiments with ellipsoid spirals made of glass. These were prepared by winding a long heat-softened glass rod (diameter: 4 mm) around the same metal rod which served for the preparation of the Chromel "A" spirals. Here, however, the turns of the spiral were spaced immediately, since after the glass cools, a readjustment by stretching is, of course, impossible. The length of these glass spirals was again 50 mm, but they comprised only 10 rings, because the diameter of the glass rod was much greater than that of the Chromel "A" wire. With the glass spirals we took the additional precaution of heat-polishing both free ends and turning them inward towards the center to prevent skin damage.

The *corkscrew spirals* were prepared essentially in the same manner except that a thicker (No. 9) Chromel "A" wire was employed whose diameter approximated that of our glass rod. However, in this case, the free ends were not turned to give a tapering ellipsoid shape to the object, but the spiral was cut so that both free ends came to lie on the

same side, which upon implantation was turned towards the back of the animal, where it could do no harm, not towards the skin, which might have been perforated. One free end was sharpened to a point on a Carborundum saw to facilitate its penetration when inserted under the skin with a corkscrew-like motion in a manner to be described later. Similar corkscrew spirals were also prepared out of the same type of glass rod that we used for the manufacture of ellipsoid spirals (Fig. 1).

Surgical procedures.—These will be described here in relation to the experimental groups as listed in Table I and discussed in the subsequent text.

In Group 1 a simple "pneumoderma," or subcutaneous airpouch, was prepared, under light ether anesthesia, by the injection of 25 ml of air under the shaved skin of the back through a gauge 27 needle. In this location the connective tissue layers are so arranged that it is quite easy to prepare a single ellipsoid air cell, the wall of which represents the simplest type of an internal wound surface.

In Group 2 the procedure was the same, but gradual collapse of the cavity was prevented by the injection of 25 ml of air twice weekly.

In Group 3 a similar subcutaneous pouch was produced in the same manner, but immediately afterwards, the air was removed and replaced by 25 ml of water (given here, as in all other groups, in the form of Ringer-Locke solution). Even in such experiments, in which the aim is to obtain a connective-tissue space filled with water, it is necessary to prepare a pneumoderma first; otherwise the injected aqueous solution merely produces diffuse edema, not a single cyst-like cavity.

In Group 4 the procedure was the same as in Group 2, but only half of the originally injected air was withdrawn and replaced by water, so that eventually the pouch contained 12.5 ml of air and 12.5 ml of water.

In Group 5, after preparation of the standard 25 ml pneumoderma, a small skin incision of about 2 cm was made in the flank region somewhat ventrad from, and parallel to, the base of the air-pouch. Through this incision the pouch itself was opened and an ellipsoid Chromel "A" No. 18 spiral was introduced into it. This is made feasible by holding the spiral compressed to a flat ring with a pair of anatomical forceps. The experiment was repeated several times also

with the stiff Chromel "A" No. 9 and glass spirals, which cannot be compressed but have to be introduced through a somewhat larger incision from the caudal pole of the pouch. In all these cases, immediately after introduction of the spiral the skin wound was closed airtight with No. 11 Michel clips and a covering layer of collodion. Then the pneumoderma was restored to its original volume by the injection of 25 ml of air through a No. 27 needle. The results in this group, as in all others, were essentially similar, whether Chromel "A" No. 18, No. 9 or glass spirals were used. However, the thin (No. 18) Chromel "A" spirals gave the most consistent results; hence, our data are derived from observations with these, unless otherwise indicated.

In Group 6 the procedure was the same as in Group 5, except that the air (which normally tends to be absorbed so that suction develops within the spiral after about 5 to 10 days) was replaced by re-injection of 25 ml, twice weekly.

In Group 7, in which compression of the spiral by suction was to be obtained, we could use only the thin, flexible Chromel "A" No. 18 spirals. The procedure was the same as in Group 5, except that immediately after introduction of the spiral and airtight closure of the wound, all the air within the cavity was removed through a No. 27 needle; thereby, the spiral was compressed to a flat ring owing to the suction exerted by the vacuum under the skin.

In Group 8 the procedure was the same as in Group 7, except that the air evacuated by suction was immediately replaced by water.

In the last two groups of the Table the procedure was the same as in Group 8, but corn oil (Group 9) or liquid paraffin (Group 10) was employed to fill the pouch, instead of Ringer-Locke solution.

In addition to the ten basic groups listed in Table I, we also performed some additional experiments with corkscrew spirals. These can be introduced into connective tissue without creating a cell-free dead space in the lumen within the coils. Here, we first infiltrated an area, somewhat exceeding the size of the spiral, under the skin of the back with 15 ml Ringer-Locke solution. Great care was taken to distribute the fluid diffusely, without injecting too much at any one place, so as not to disrupt, but merely to distend, the normal connective-tissue lattice. Then into this edema-pad the corkscrew spiral was introduced (pointed end first) through a 2 mm incision by turning the object around its axis in a corkscrew-like fashion (Fig. 1). In

the edematous, distended subcutis this is quite easily possible without much injury to the skin. A single No. 11 Michel clip and a drop of collodion sufficed to close the minute skin opening through which the spiral was introduced. The same procedure was employed both for glass and for Chromel "A" No. 9 corkscrew spirals. Spirals made out of Chromel "A" No. 18 do not lend themselves for this use, because they are too thin and tend to bend while being screwed through the skin.

FIGURE 1
INTRODUCTION OF A GLASS CORKSCREW SPIRAL

Observations and measurements.—The air and water content of the pneumodermas could readily be checked day by day in a dark room by transillumination with an electric flashlight, which permits the accurate determination of the fluid-level within the pouch. The same technique may be employed for the estimation of the speed of liquid absorption in subcutaneous spirals. On the 30th day all animals were

killed with chloroform, and the pouches and spirals macroscopically examined. Subsequently the tissue within the scaffoldings was fixed in Susa solution and embedded in paraffin for the microscopic examination of sections stained with hematoxylin-phloxine or van Gieson's technique.

Results

The principal results of our experiments are summarized in Table I.

Air is completely absorbed from a cavity lined by connective tissue, such as the lumen of the pneumoderma (Group 1). The absorption is rapid, usually being terminated within ten days or so. By the 30th day no trace of the original pneumoderma could be detected even upon histologic examination of the injection sites. Even if the pneumoderma pouch was maintained by weekly re-injections of air (Group 2) there was neither exudation of fluid nor proliferation of the parietal connective tissue.

TABLE I
Organization of Various Gaseous, Aqueous and Solid Tissue Scaffoldings

Group	Contents of the Pneumoderma		
	1st day	2nd day	30th day
1	Air	Air	0
2	Air (maintained)	Air	Air
3	Water*	0	0
4	Air + Water*	Air	0
5	Air + Spiral	Air	Exudate + Fibrin
6	Air (maintained) + Spiral	Air	Air
7	Spiral (with suction)	Exudate	Exudate + Fibrin + Connective Tissue
8	Water* + Spiral	Water + Exudate	Connective Tissue
9	Oil + Spiral	Oil	Oil
10	Paraffin (liquid + Spiral)	Paraffin	Paraffin

* Administered in the form of Ringer-Locke solution.

If water was substituted for air in the pneumodermas, the contents were completely absorbed within a day; indeed, even after one or two hours no significant water residue could be detected in the animals so treated (Group 3). Since, here, parietal fibroplasia was not stimulated by the fluid, the site of the intervention could no longer be detected at the time of autopsy on the 30th day. In pneumodermas filled originally with air and water in equal proportions, the water was absorbed within the first few hours, while most of the air was still present on the second day and disappeared only gradually in the course of the following week (Group 4).

The results were altogether different if the collapse of the pneumoderma was prevented by the insertion of a spiral (Group 5). Here, the absorption of the air proceeded as in the previous groups until the skin became tightly attached to the artificial scaffolding, then exudation and fibrin formation commenced. At the end of the experiment the lumen of the spiral was completely filled with a transparent, slightly yellowish, exudate and some fibrin filaments developed along the axis of the spiral. Apart from the formation of a thin capsule that enveloped the spiral, fibroplasia was virtually absent.

Spirals kept in pneumodermas in which the lumen was maintained by constant re-insufflation of air (Group 6) remained unattached. Here, there was no trace of exudate, fibrin, or connective-tissue development, even after 30 days.

When the spirals were collapsed, by aspiration of all the air that was originally present in their lumina (Group 7), exudation began almost immediately and within a few hours after the operation the spirals became partially extended again owing to the gradual diminution of suction. Under these conditions, after 30 days, the lumina of the spirals were filled not only with exudate and fibrin, but also with considerable amounts of loose connective tissue. The latter formed a tube just inside the coils, but invariably still left a large axial space which did not become organized by connective tissue, but contained only exudate and fibrin.

When the spirals were placed in pouches completely filled with water (Group 8), the fluid outside the limits of the spiral was absorbed in a few hours, but the space within the coils remained filled with fluid in which yellowish exudate gradually replaced the original Ringer-Locke solution. The most noteworthy observation in this group was the eventual complete replacement of fluid by loose gelatinous connective tissue, which totally filled the scaffolding by the 30th day (Fig. 2).

Corn oil (Group 9) or liquid paraffin (Group 10) could not substitute for the Ringer-Locke solution under similar circumstances: these fluids did not promote connective-tissue growth nor were they absorbed.

Numerous additional experiments were then performed under essentially similar conditions with glass and metal spirals of different length (1-10 cm) and width (1-3 cm), but these will not be reported here

FIGURE 2

ELLIPSOID CHROMEL "A" NO. 18 SPIRALS ON THE 30TH DAY AFTER IMPLANTATION

A: Scanty fibrin threads in a spiral in Group 5 which was originally filled with air. The empty space between the coils was filled with exudate.

B: Cross section through contents of the scaffolding shown in *"A."* Acellular fibrin threads. (v. Gieson, X 100).

C: A similar spiral in Group 8. Here, the air was replaced by water immediately after implantation and the entire lumen of the scaffolding is completely filled by loose connective tissue.

D: Cross section through contents of the scaffolding shown in *"C."* Fully organized loose connective tissue. (v. Gieson, X 100).

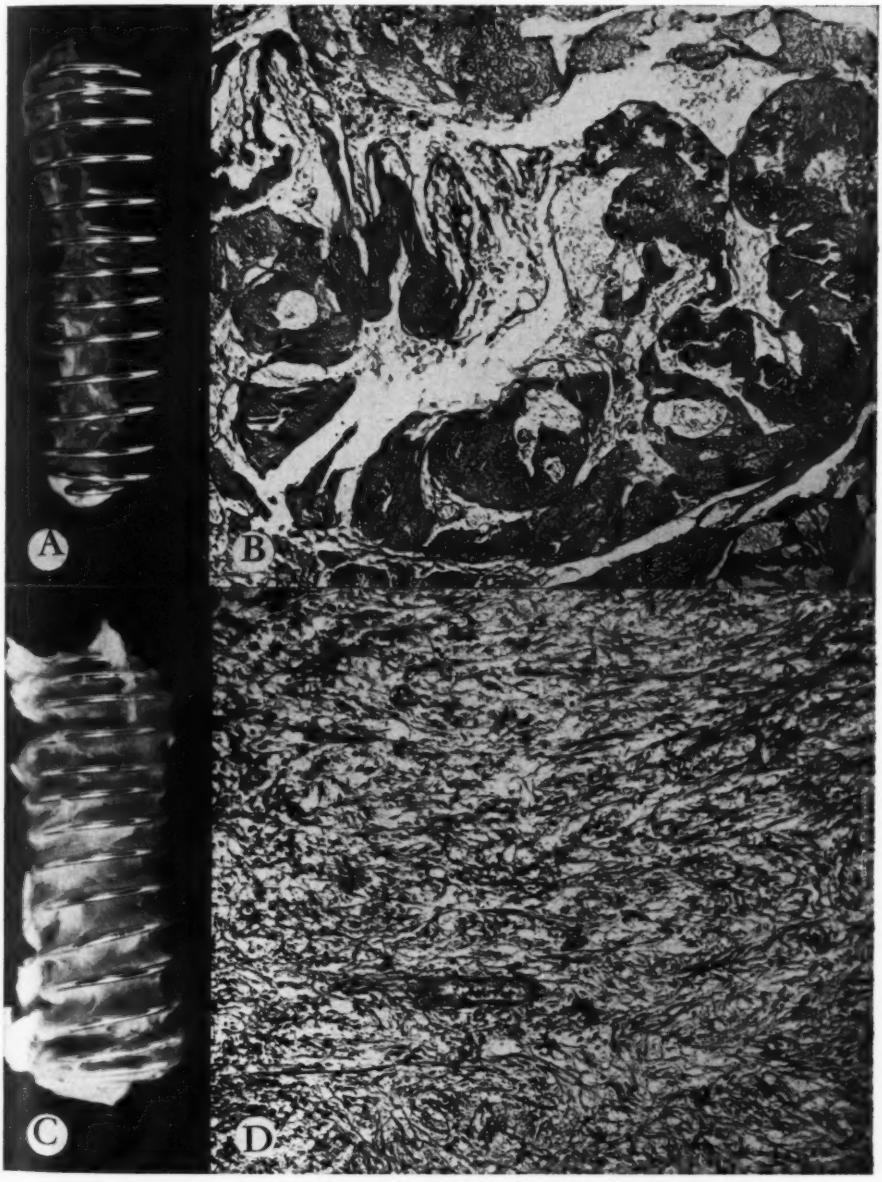
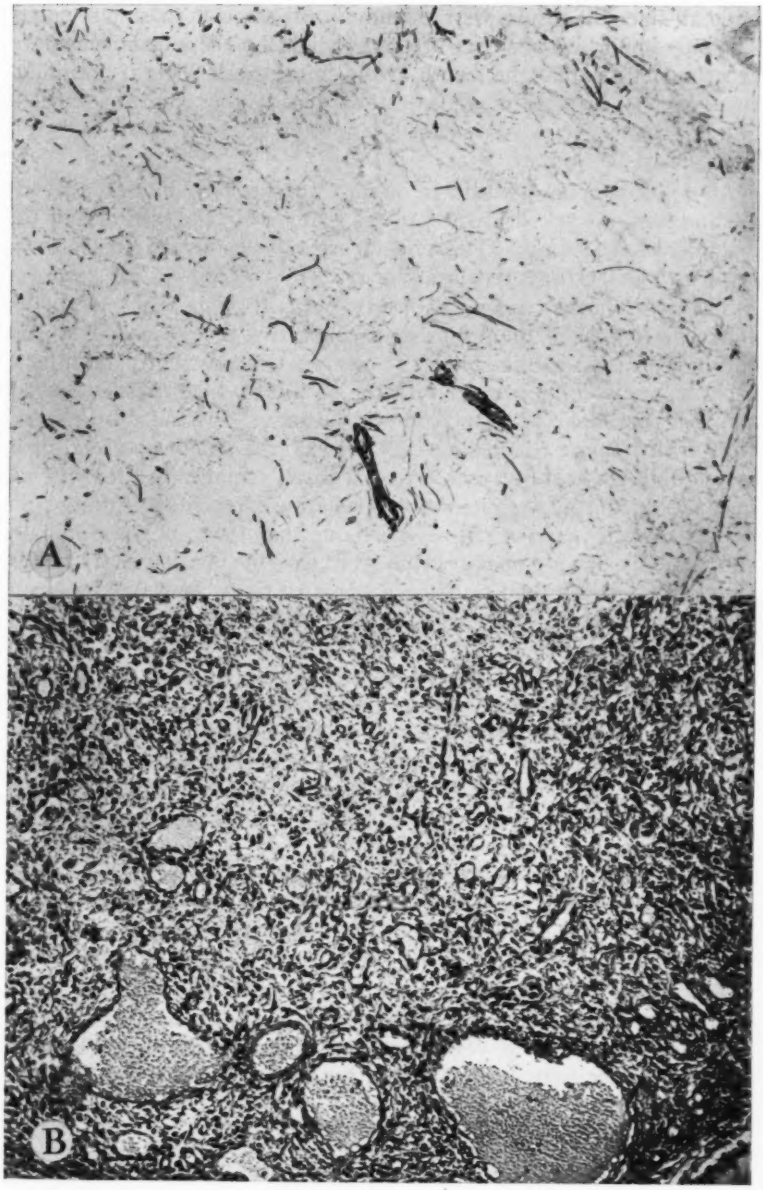

A
B
C
D

FIGURE 3

STIMULATION OF CONNECTIVE-TISSUE PROLIFERATION IN THE CORKSCREW-TYPE OF SPIRAL

A: Marked edema of the connective tissue around two small blood vessels, immediately after insertion of the spiral.

B: A similar region after five days shows intense fibroblast proliferation (without the formation of a fibrinous preliminary scaffolding) and pronounced vascular dilatation. (v. Gieson, X 100).


A
B

in detail since the results were essentially the same as those just noted. The only important new fact brought to light by these complementary experiments was that the length of the spiral exerts little if any influence upon the speed of organization, but if the diameter exceeds 20 mm, connective-tissue ingrowth is always extremely slow and usually non-existent.

Apparently, connective tissue ingrowth occurs only into an aqueous medium and the speed of the process is inversely proportional to the diameter of the gap, that is, the distance between the pre-existent connective-tissue surfaces that are separated by the coils.

The question then arose whether the rate of repair could be greatly enhanced if some of the pre-existent connective tissue were left intact within the lumen of the spiral. To explore this possibility, additional experiments were performed with the corkscrew-type of spiral which makes it possible to surround pre-existent tissue (distended by edema) with an inanimate scaffolding that prevents its collapse. Here again metal and glass spirals yielded essentially similar results. In all instances histologic examination immediately after introduction of the spirals showed that the edematous tissue that they enclose originally consists only of very sparse fibers, cells and blood vessels. However, substitution of the fluid by loose connective tissue had already begun after three days and was virtually complete within a week (Fig. 3). Here again the presence of an aqueous medium proved to be quite indispensable for cell proliferation. If immediately after introduction of a spiral into the subcutaneous edema-pad 15 ml of air is injected at both ends of the foreign body, the suction, which normally fixes water within the spiral, can be abolished. In this event, the extremely loose, pre-existent connective-tissue framework in the lumen (in which the fluid medium is now replaced by air) shows no signs of active proliferation.

A final experiment was then designed to determine whether, during complete starvation, water fixation in a limited area could produce topical tissue growth at the expense of other parts of the body. Both glass and metal spirals of the corkscrew-type were introduced into subcutaneous edema-pads as usual, but immediately after the operation all food was withdrawn. Within 5 days the animals lost 25% of their body weight and yet edematous connective tissue proliferated virtually at the same rate as in control animals, so that the lumen of

the spirals became almost completely organized by the 5th day (Fig. 4).

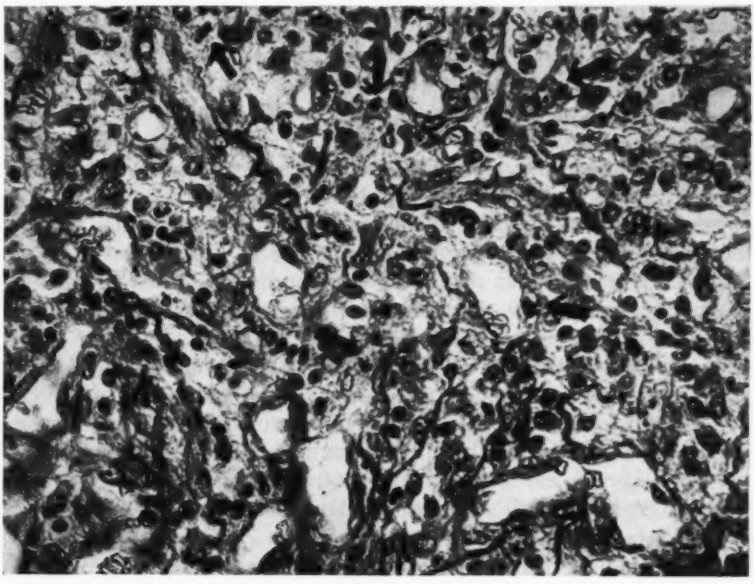


FIGURE 4

TISSUE PROLIFERATION IN A CORKSCREW-TYPE OF SPIRAL DURING FASTING

This animal received no food after the implantation of the spiral during five days and although it lost 27% of its body weight, there is active mitotic proliferation of fibroblasts (arrows) and neoformation of fibrils. Many of the fibrocytes are unusually large and intensely basophilic. (v. Gieson, X 400).

DISCUSSION

The experiments just reported show that fixation of water, in itself, can act as a potent stimulus for connective-tissue growth.

The findings summarized in Table I indicate that a wound—an artificially produced severance of tissue continuity—is not, as has often been thought, necessarily the stimulus for the initiation of wound healing: no sign of connective-tissue proliferation and cicatrization was observable in the walls of ordinary air pouches of the pneumoderma type. This was so irrespective of whether the originally

introduced air was allowed to be gradually absorbed or whether the internal wound surface was kept gaping by repeated air insufflations (Groups 1 and 2). It may be objected that, under these conditions, contraction of the pouch is physically impossible because the cavity is distended with air. This argument is not valid: our earlier observations (1) have amply demonstrated that as soon as an irritant (e.g. croton oil) is introduced into a pneumoderma, concurrently with the initiation of exudation, the wall of the air cell is transformed into a granuloma which shows intense contraction. The contracting pouch merely compresses the air and thereby accelerates its absorption. The conclusion appears to be unavoidable, therefore, that neither the production of a wound surface nor its exposure to air necessarily stimulates tissue growth and repair. It remains to be proven whether inflammatory irritants become active in this respect because they provide an aqueous medium by exudation. The exudate that fills the cavity of a granuloma pouch is not a suitable medium for the ingrowth of connective tissue, presumably because of the previously mentioned unfavorable metabolic conditions (accumulation of enzymes and acids, high osmotic pressure) that prevail within inflammatory fluid. Still the filtration of less contaminated water through the capsule may account for the growth of connective tissue in the granulomatous wall itself.

If the air in a pneumoderma is completely (Group 3) or partially (Group 4) replaced by water, there is no stimulation of connective-tissue proliferation but, since the injected fluid is rapidly absorbed, the possible effects of water fixation could not become manifest.

The presence of a chemically inert foreign body, such as a spiral of Chromel "A" or glass, likewise failed to induce inflammatory exudation or connective-tissue proliferation in the pneumoderma as long as air was present in the cavity (Group 6), but if the air was allowed to be absorbed (Group 5), then inflammatory exudate and fibrin gradually took its place. This was probably the consequence of the suction that develops within such spirals as their gaseous content disappears. Yet, even here connective-tissue proliferation was absent or minimal, presumably because of the previously mentioned accumulation of toxic metabolites in inflammatory exudates.

If suction is produced within a spiral by aspiration of the air (Group 7), the cavity rapidly fills with plasma filtrate. In this fluid

some connective-tissue proliferation is possible perhaps because, at least at first, it does not yet contain any significant amount of toxic metabolites. In any event, tissue growth within such structures is minimal.

The situation is altogether different in spirals which are completely filled, from the start, with a metabolite-free aqueous medium, such as Ringer-Locke solution (Group 8). It is here that we find the most rapid organization of the lumen by loose connective tissue. This effect of water is rather specific in that other non-irritating fluids, such as corn oil (Group 9) or liquid paraffin (Group 10), cannot replace water as a medium for connective-tissue growth. Of course, it may be argued that it is not the presence of water that stimulates tissue growth in a cavity, but the presence of any filler other than water that prevents it. However, the two formulations express the same thought: only water can act as a growth-stimulus.

The experiments with the corkscrew-type of spirals further substantiated the view that whenever water, free of injurious substances, can be fixed in connective tissue, topical growth is enhanced. In addition, the experiments demonstrated that this process is further facilitated by the presence of at least some loosely arranged, pre-existent connective-tissue elements within the cavity that is to be populated by a newly-formed tissue. In fact, we saw that even during starvation, when there is catabolism in other parts of the body, the fixation of water in a limited connective-tissue area could still induce topical growth.

We may ask why, in clinical conditions conducive to edema, water retention does not produce fibroplasia more regularly. It must be remembered, however, that under pathological conditions edema does not tend to occur without simultaneous local interference with tissue metabolism and nutrition, that is without creating conditions inimical to cellular growth. This is the case for example in cardiac, toxic and inflammatory edema as well as in lymphedema. Besides, even under these circumstances some connective-tissue proliferation does quite frequently occur, especially in chronic lymphedema, the condition that is perhaps most comparable to the experimental situations which we created.

Of course, water being the matrix of all living tissues, it required no proof that growth, or even life itself, is impossible without it. By

way of an introduction, we also mentioned that growing tissues are notoriously rich in water. It is self-evident, therefore, that growth must be accompanied by water fixation. Our observations suggest, however, that water fixation in tissues not only accompanies, or even precedes, but actually *causes* growth, since tissue proliferation followed when nothing was changed in an area except for the introduction of water. Apparently, the provision of an aqueous milieu is not only a prerequisite, but—at least within limits—the actual cause of cell proliferation.

Is this concept applicable to all tissues? Our work was wholly limited to the study of subcutaneous connective tissue; it is not justified, therefore, to generalize at this time. Whether water fixation could have similar effects upon other tissues cannot be determined by reasoning but only by additional experiments on various organs. It may be worth mentioning, however, that some earlier observations seem to be compatible with such a generalization. For example, during periods of rapid growth, an increase in water content occurs, not only in connective tissue, but also in various epithelial organs. The structural changes that take place in the most characteristic organ of longitudinal growth, namely the junction-cartilage disk, are also compatible with our concept. Here, the chondrocytes that are arranged in columns as a preparation for endochondral bone-formation swell enormously until their capsule bursts. Then new growth takes place in the remaining rigid, fluid-containing cavity, which is essentially a miniature replica of the kind of scaffolding that we have placed artificially into the subcutaneous tissue.

In conclusion we must place special emphasis upon the fact that actually we showed only that water fixation *can* act as the ultimate cause of connective-tissue growth. It remains to be seen whether it *always is* functioning in this capacity and whether it also does so in structures other than connective tissue. Be this as it may, our technique permits the fixation of water in circumscribed regions without producing any associated injurious metabolic derangements, such as result from inflammation or venous and lymphatic stasis. Using this technique, we believe to have shown that water fixation, in itself, can act as the ultimate cause of connective-tissue growth.

Summary

An experimental technique has been developed for the fixation of large quantities of water in subcutaneous tissue without producing any marked local metabolic derangements such as occur in toxic and inflammatory edema or water retention due to interference with the blood supply and lymphatic drainage.

The technique consists in the introduction of metal or glass spirals into the subcutaneous connective tissue of the rat, the space enclosed by the coils being filled with an aqueous Ringer-Locke solution. Under these conditions the water cannot be rapidly absorbed—as it would be after injection into ordinary, or air-distended, connective tissue—because it is retained by the negative pressure that develops within such tissue-scaffoldings.

Using various modifications of this technique, it has been shown that water thus fixed is rapidly replaced by loose connective tissue. Control experiments revealed furthermore that, in such a tissue-scaffolding, the lumen cannot be similarly organized if it is filled with air, corn oil or paraffin. It is concluded that, here, the fixation of water itself acted as the ultimate cause of connective-tissue growth.

These observations raised the question whether, in the final analysis, tissue growth might not be dependent everywhere upon water fixation; this possibility is discussed on the basis of known data on water distribution and swelling in actively growing organs.

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BOOK REVIEW

METHODS FOR RESEARCH IN HUMAN GROWTH. Stanley M. Garn and Zvi Shamir. Pp. xi 121. $4.75, Springfield, Ill., Charles C Thomas, 1958.

In their introductory chapter, the authors state that the objective of their monograph is to provide a guide to methods, rather than a Handbook of Growth Research, and add that their book is not devoid of opinions. This latter is a fortunate decision for the reader, because admirable though their summary of methods and their description of techniques are, the authors' critical evaluation of them will be of even greater service to those who plan either a comprehensive study of human growth and development or the exploration of only some limited aspect of that field. For the former, the chapter entitled, *Planning the Study and Selection of Subjects,* contains much practical and, therefore, useful information based on many years of first-hand experience in conducting such an enterprise. Those who permit themselves to be guided by the admonitions which it and the subsequent chapters contain will avoid the error of adopting procedures and techniques which the experience of others have shown to be of little or no value.

Those who are familiar with the multiplicity of the anthropometric measurements that were made in some of the early growth studies in this and in other countries and who are aware of how little most of those measurements added in the way of significant information about the growth of children will applaud the statement on p. 35: "As a general rule the fewer the anthropometric measurements, the better." The authors suggest those measurements which they believe are of value and state quite convincingly their reasons for their opinion.

In addition to chapters devoted to various morphological aspects of human growth and development, there are others which deal with physiological measurements, biochemical tests, and statistical analysis and graphic representation of growth data. The book's value is further enhanced by an adequate index and a very useful list of references.

The reviewer knows of no other book which so adequately covers this subject and very few on any subject in which compendiousness and clarity of expression are so happily combined. Garn's and Shamir's monograph will be very useful to pediatricians and other physicians who deal with children as well as to those who are actively engaged in studies of growth and development.

WILLIAM WALTER GREULICH